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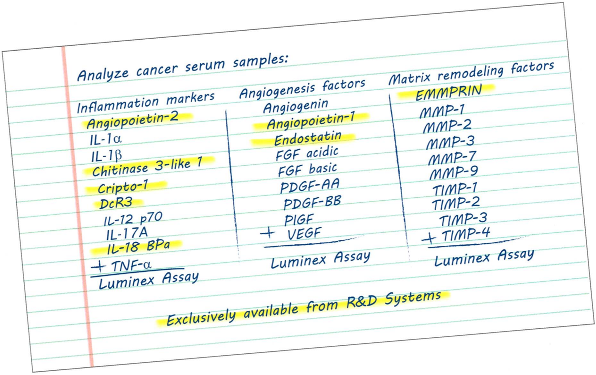

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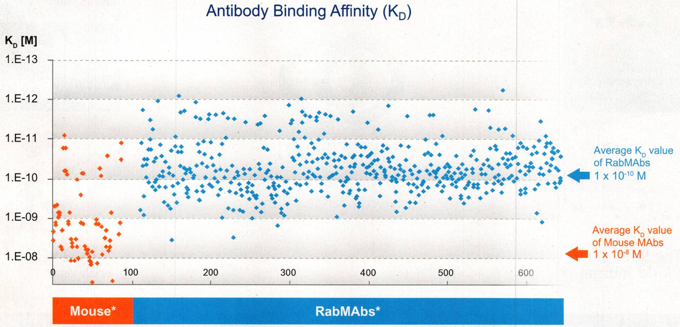

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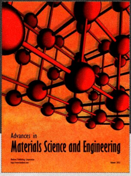
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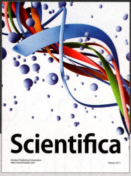
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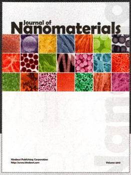
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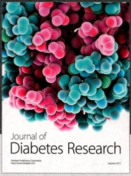
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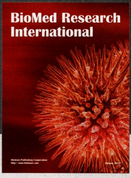
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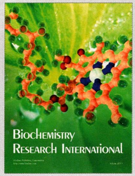
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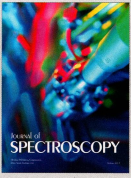
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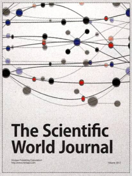
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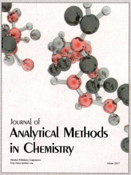
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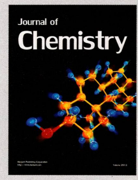
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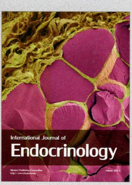
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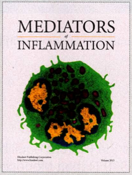
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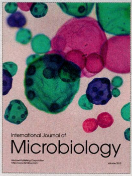
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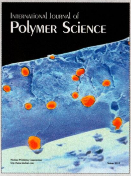
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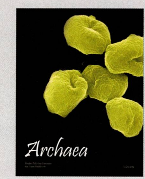

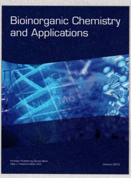
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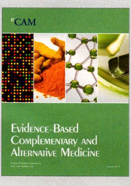
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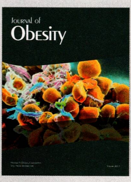
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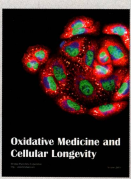
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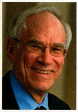

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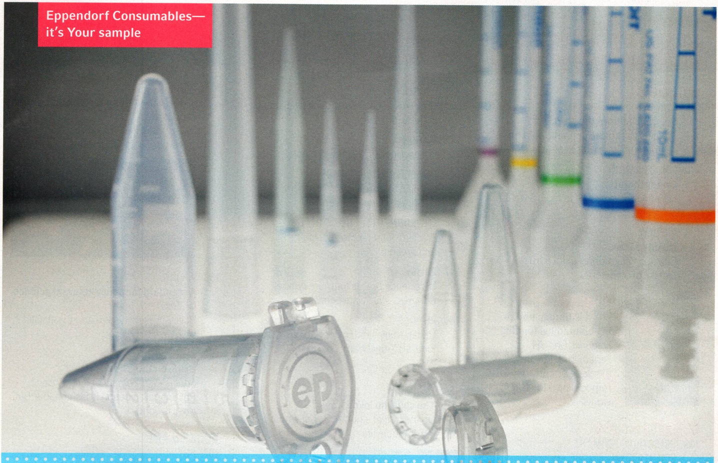

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THIS WEEK

EDITORIALS

MEMOIRS The pleasure and pitfalls of a written scientific life **p.136**

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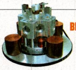

BIG-G What goes up must come down slightly faster **p.139**

Sequenced from the start

Four US studies are set to explore how genomic data can best help healthy and ill newborns. They must also settle some questions of ethics.

Genetic sequencing has established itself as a powerful tool for diagnosis, but it is not yet clear how useful it will be for disease prevention or health management. A US$25-million project announced last week aims to explore that issue in perhaps the most high-stakes patient group: newborn babies.

In the Genomic Sequencing and Newborn Screening Disorders (GSNSD) programme, four teams will sequence the exomes — the protein-coding portions of the genome — or the whole genomes of more than 1,500 babies, including not only infants who are ill, whether or not the disease has been diagnosed, but also healthy babies. The programme is funded by the US National Human Genome Research Institute and the Eunice Shriver Kennedy National Institute of Child Health and Human Development (NICHD). The studies will examine how useful sequencing information is for families and doctors, and whether it is superior to data gathered through conventional newborn-screening methods, which check for about 60 genetic disorders.

The project joins a short but growing list of studies testing the utility of clinical sequencing in otherwise healthy individuals, but it is the first to focus on healthy and ill babies. As such, it will highlight five hot questions.

First, do we yet know enough about how genes code for health to make genomic data useful in preventing disease? Studies have found that sequencing can diagnose 15–50% of children with otherwise undiagnosable illnesses, but no one has yet asked what use it has for healthy children. Not all genetic traits will influence a person's health, and it is still not possible to say with any certainty what a given genetic variant will mean for a given individual.

Second, what kind of genetic findings should doctors return to patients, and does the answer differ between children and adults, or between ill and healthy people? The family that is unsure whether its ill baby will live or die is not in a good position to absorb information about a hypothetical future cancer risk. The family that has a baby's genome sequenced just to see what might be found may spend years worrying about that cancer risk in their perfectly healthy child. The key will be to find the children who will best benefit from this knowledge, because their individual disease risks are real enough that routine screening could save their lives. In the US health-care system, which is prone to over-diagnosis and over-treatment of cancer, for example, this is a tricky balance. Some of the GSNSD projects will check babies' genomes for genes not linked to any immediate illness, although each study is taking a different approach to how it will inform parents about risks.

Third, what is the quickest and cheapest way to conduct clinical sequencing so that it returns accurate information to patients in time to influence care decisions? Increasing the number of conditions being screened for will necessarily cause more false positives — as many as 20 for every true positive, according to one estimate. Those false positives will lead to increased medical costs and anxiety for families.

Fourth, who owns the genetic data? None of the GSNSD studies plans to give the raw genetic data to the children's families, even though that could allow the children to benefit from it throughout their lives.

Finally, should the data be shared with other researchers? This would be the best way for scientists to help tackle the tough question of how genes contribute to disease. But it is increasingly difficult to guarantee the privacy of genetic data (see *Nature* **493,** 451; 2013), and this is an important issue for babies, whose information will be known for their entire lives even though they themselves have not consented to the disclosure. One of the GSNSD projects will share data with the NICHD's Newborn Screening Translational Research Network, and another with the National Center for Biotechnology Information's Database of Genotypes and Phenotypes. The other two are still deciding.

> ***"The day when all children will be sequenced at birth — if not before — draws ever nearer."***

As researchers explore these questions, sequencing costs continue to drop and the day when all children will be sequenced at birth — if not before — draws ever nearer. Some people are wary of this, and are already warning of the dangers of what they consider to be a government-funded plan to store all citizens' data. If newborn sequencing is to fulfil its potential to save many children's lives, it is imperative that scientists get the ethics and the science right. ■

Under threat

The grey wolf is at risk of losing its endangered status under US law.

In the mid-1990s in the United States, several courts were asked to decide whether wolves were illegal immigrants. Ranching groups that were against the proposed reintroduction of the wild animals to Idaho, Wyoming and Montana were trying to block their transport across the border from Canada. The appeal failed and the foreign wolves were delivered and released.

In recent decades, many more in the legal profession have become familiar with *Canis lupus*. The grey wolf, and its place in the US landscape, sharply divides opinion — both scientific and political. Broadly speaking, conservationists want the wolf population to expand into its historical range, whereas the ranching community is anxious about large numbers of a top predator roaming free. Both sides can point to scientific research and ecological opinion to support their stance.

The battle over the fate of the grey wolf is gearing up for a new conflict, perhaps the most significant yet. As we report on page 143, the US Fish and Wildlife Service (FWS) has extended the period for which it will allow public comment on its controversial proposal to strip the grey wolf of its federal protection. The agency wants to remove the animal from the list of those protected under the 1973 Endangered Species Act, and to hand responsibility for its management and conservation to individual states. The FWS claims that the move follows the "successful recovery" of the wolf in two key regions. Scratch the surface, though, and it looks more like a cost-cutting exercise and, to some, a politically convenient one. Gary Frazer, assistant director for endangered species at the FWS, told *Nature* that he expects hundreds of thousands of people to comment.

Many of these will be rightly suspicious of the true motives. The proposed delisting of the grey wolf comes barely two years after the notorious 'wolf rider' that saw a clause to remove legal protection of the animals in Montana and Idaho tagged by local politicians to an unrelated, and essential, budget appropriations bill. The move, this journal noted at the time, set a "dangerous precedent" (see *Nature* **475,** 5; 2011), and was the first time that Congress had removed a species from the list. The clumsy political manoeuvre came after a decade of court-rebuffed attempts to change the status of the wolf through the proper regulatory channels.

Grey wolves are certainly doing better in the United States than a century or so ago, when rewards for their killing made them locally extinct. Controlled reintroduction under the 1973 act has led to populations in the thousands around the Great Lakes and Northern Rockies — and to the loss of livestock. According to the US Department of Agriculture, between 1995 and 2007, wolves killed 298 cattle, 46 sheep, 13 llamas, 24 goats and 7 horses in Montana. Enough is enough, critics say; the grey wolf is no longer endangered. Yet the 1973 act is clear: such a judgement must be made over all, or a significant proportion, of the animal's range.

"We still haven't figured out how to handle a situation where experts have outspoken views," Frazer says of the divergent opinions on the topic. It is a lament that will strike a chord with many policy-makers, not least those in Britain, where government-sanctioned marksmen are busy reducing the population of another emblematic — and previously protected — species. After years of similar arguments and conflicting scientific advice, the environment department DEFRA has embarked on two pilot culls of badgers, which farmers blame for the spread of bovine tuberculosis (TB).

Ian Boyd, science adviser to DEFRA, writes on page 159 that, too often, the evidence used to set policy is biased and unreliable, even when published in scientific journals. He wants to introduce a Kitemark (indicator of quality approval) for studies that meet an audited standard of scientific evidence. But he should be careful what he wishes for. As *Nature* has repeatedly pointed out, the published evidence on badger culls does not indicate that bovine TB will be reduced by DEFRA's strategy, which relies on untested tactics such as free shooting.

"Enough is enough, critics say; the grey wolf is no longer endangered."

Politics can trump science, of course, for politicians are elected to make decisions. But so can sentiment. A few miles along the M4 motorway from where the badger culls and the protests against them are under way is the base of the Wolf Conservation Trust. Wolves vanished from Britain centuries ago, but they retain mystique and appeal — even to Brits. US lawmakers must bear this in mind as they invoke science to argue for the delisting of the grey wolf.

The protection of the 1973 Endangered Species Act for vulnerable animals does not end at the US border. Several overseas and foreign species are listed too, and US citizens are forbidden from, for instance, trading in them. But a US law that gives sanctuary to the Chinese alligator and the great Indian bustard but not to the native grey wolf would be a strange beast indeed. ■

Reality at risk

Don't treat a memoir as anything other than one person's interpretation of events.

"It was the afternoon of my eighty-first birthday and I was in bed with my catamite when Ali announced that the archbishop had come to see me." So runs the first sentence of *Earthly Powers*, Anthony Burgess's memoir of the fictional novelist Kenneth Toomey. "I have lost none of my old cunning in the contrivance of what is known as an arresting opening," he writes a few lines later — noting that, whereas every supposed fact in the first sentence is true, the context is one of pure artifice, designed to portray an image of the writer as he would wish to be seen, not necessarily as he really is. Toomey is clearly a writer of some skill (as is Burgess, his inventor and rapporteur), so we, the poor readers, are at his mercy. We can do no other than take what he claims as truth at face value, whether it is true or not. Such is the *caveat emptor* of the memoir in general.

This week, *Nature* publishes book reviews of two memoirs by very real people. On page 162, Robert Crease reviews *My Brief History* by theoretical physicist Stephen Hawking, and notes that it is more akin to a PR exercise than a warts-and-all confession: "It does not take the reader behind any scenes … It is a concise, gleaming portrait, not unlike those issued by the public relations department of an institution." Eugenie Scott on page 163, by contrast, finds *An Appetite for Wonder*, the first volume of memoirs by evolutionary biologist Richard Dawkins, "a very honest book", in which we get a taste of the upbringing and early experiences of the author of *The Selfish Gene* and *The God Delusion*. From which we are entitled to get a flavour, at least, of how Dawkins got to be the man he is today — in other words, what makes him tick.

But do we? Get a flavour of what makes someone tick from their own, self-selected, self-redacted reminiscence? Memoirs are more vehicles of entertainment than any reflection of reality. When one reads *The Double Helix*, James Watson's knockabout account of the discovery of the structure of DNA, one should take any facts presented therein strictly as having been heavily filtered by the unashamedly biased reminiscence of just one of the protagonists, not as a scholarly account. And there's nothing wrong with that. *The Double Helix* works beautifully as entertainment.

There is another layer of selection. Those memoirs that get published as books are not so much about scientists (say) as celebrities. Readers of *My Brief History* will want to know about Hawking's triumph over his disability more than how he came to this or that conclusion about black holes. More people are likely to have encountered Dawkins as the doctrinaire neo-atheist of *The God Delusion* than as the peerless commentator on the machinery of evolution in *The Selfish Gene* — and vastly more than as the author of scientific papers on animal behaviour.

To understand what working scientists free from the constraints of celebrity actually do all day, one might turn to the blogosphere and follow (to mention just two of thousands) Jenny Rohn's 'Mind the gap' (http://occamstypewriter.org/mindthegap) and, perhaps more pertinently, the notes of the anonymously eponymous Female Science Professor (http://science-professor.blogspot.co.uk).

Even then, such writings demonstrate the self-selection of those scientists (a tiny proportion) who feel that they have something to say. For everyone else, life is something that is lived undocumented, unshared and in real time. Perhaps the only really 'true' experiences are those that one has lived oneself. To which one can only ask whether one is talking to oneself, or whether the Universe has gone solipsistic all of a sudden. Then again, to quote that koan from Jewish Buddhist wisdom — if there is no self, whose arthritis is this? ■

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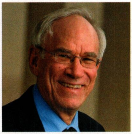
WORLD VIEW *A personal take on events*


UT SOUTHWESTERN

It is time to update US biomedical funding

The effects of federal budget cuts provide an opportunity to revisit the funding structure of the National Institutes of Health, says **Frederick Grinnell.**

As the 30 September deadline looms for US lawmakers to complete budget appropriations for the fiscal year 2014 with no realistic prospect of success, drastic cuts to public spending on science seem likely to continue. The effect has been severe: many grant-renewal applications at the US National Institutes of Health (NIH), which were easily fundable a few years ago, are not even close to being funded this year. Things are tough, as reflected by an outpouring of comment and opinion in the media about the potential decline of US science.

We have been here before. Around 1970, US spending on science was cut to help pay for the Vietnam War. Faculty positions were scarce and winning a research grant became much more difficult. But the research community survived and thrived. I believe that we will survive the current cuts, but because of their depth and arbitrary nature, their effects will take years to overcome.

We should take this opportunity to examine and debate the NIH funding structure for biomedical research, and focus on how it could be made stronger, more resilient and more diverse in the future. To begin, here are four suggestions.

First, we should revisit the relationship between how NIH grants are assessed and funded. Grant applications are evaluated on a relative scale, but are funded in absolute terms: all or nothing. This is illogical. Applications that fall on either side of the funding cut-off, or payline, are more or less of equal quality. As overall success rates decline, this practice becomes more difficult to justify. A better approach would be to link funding levels with the percentile scores used to rank applications. NIH institutes should agree on the total number of grants to be funded, then give full funding to applications with the best scores and partial funding to those with slightly lower scores.

THE NIH FUNDING STRUCTURE COULD BE **STRONGER,** MORE RESILIENT AND **MORE DIVERSE.**

If the total available funds declined, then the curve of percentage funding versus percentile score would become steeper — perhaps down to 50% of the approved budget for grants with percentile scores just within the payline. If funds increased, then the curve would flatten out. In times of severe budget constraint, this would allow more laboratories to stay open — albeit on a smaller scale — preserving research infrastructure and lab continuity. Many will argue that this would produce labs that are unable to achieve their proposed specific aims, but investigators could always drop an aim.

Second, we should look closer at research productivity and the size of grants. Some data on the scientific output of researchers funded by the US National Institute of General Medical Sciences (NIGMS) suggest that productivity (measured by number of publications or average journal impact factor) peaks when principal investigators have annual funding levels of roughly US$750,000. When the productivity metrics were normalized to grant dollars, however, smaller labs were more productive per dollar.

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The NIGMS findings are preliminary. Moreover, some types of research are much more expensive than others. However, if NIH scientific-review groups evaluating grant-renewal applications paid more attention to normalized rather than total productivity, then principal investigators with smaller labs might become more competitive for funding, resulting in a wider distribution of research dollars.

At present, when a scientist wins an NIH grant, a percentage of the funding is often used for his or her salary. In my view, that percentage is too high when faculty members are asked by their institutions to raise most or all of their own salaries through grant awards. Doing so creates both potential conflicts of commitment and challenges to research integrity. Faculty members with NIH funding bring benefits beyond the successful completion of research projects. They boost student education and the development of intellectual property and technology transfer. My third suggestion is that universities should chip in more to pay these academics, freeing up federal money for the research itself.

My final suggestion is to consider the broader impact of research grants. As pressure on public funds intensifies, scientists are increasingly being asked to articulate the economic benefits of their discoveries. Yet the economic impact of biomedical research goes beyond its ability to improve human health. It includes education of the scientific workforce, expansion of institutional and community resources, and development of regional technology centres.

Funding for academic research and technology development tends to be concentrated in a relatively small number of institutions. If 'scientific merit' remains the singular basis for decision-making on grants, then historically underfunded institutions are at a disadvantage — and the wider economic benefits cannot be shared. Yet incremental increases in funding would have a bigger relative impact for such institutions than for those already receiving the most research dollars. Perhaps grant applications from underfunded institutions should be funded at a different payline. That would be controversial but there is precedent: the NIH has already modified the payline to increase funding for new investigators, another underfunded group.

These are important points to debate, and some will be unpopular. The last consultation effort with the broad US biomedical community was in 1992, when I and many others served on the NIH Strategic Plan Task Force. That failed because the process was too top-down. We should try again. ■

Frederick Grinnell *is in the Department of Cell Biology at the University of Texas Southwestern Medical Center in Dallas.*
e-mail: frederick.grinnell@utsouthwestern.edu

RESEARCH HIGHLIGHTS

Selections from the scientific literature

CHEMISTRY

Power from deep-sea vents

Researchers have harnessed deep-sea hydrothermal vents to produce electricity.

Masahiro Yamamoto of the Japan Agency for Marine-Earth Science and Technology in Yokosuka and Ryuhei Nakamura of the RIKEN Center for Sustainable Resource Science in Wako and their team exploited the differences in chemistry between sea water and fluids that leak from hydrothermal vents. Steep concentration gradients of chemicals such as hydrogen sulphide allowed the researchers to generate more than 21 milliwatts of power from a fuel cell based on a platinum cathode and an iridium anode.

This successfully powered three light-emitting diodes 1,000 metres below the surface, both at an artificial vent created by deep-sea drilling and a natural vent.

Angew. Chem. **http://doi.org/f2dtrm (2013)**

GEOLOGY

Meet the world's largest volcano

A submarine mountain in the northwest Pacific could be the largest single volcano on Earth, rivalling even the mighty Olympic Mons on Mars.

Tamu Massif, situated 1,500 kilometres east of Japan, is roughly the size of the British Isles. Seismic-profiling studies penetrated its depths and revealed that its lava flows dip away from the volcano's summit in all directions. This suggests that all the lava came from a single eruptive vent, say William Sager of the University of Houston in Texas and his colleagues.

The dome-shaped mountain probably formed in a single eruptive blast that began some 140 million years ago.

Nature Geosci. **http://doi.org/nqd (2013)**

For a longer story on this research, see go.nature.com/beeqp4

HENNO GOUS

ANIMAL BEHAVIOUR

Clumping caterpillars

Entomologists sometimes see caterpillars clumping together, but the reason for this behaviour has defied explanation.

Researchers had proposed that the aggregations conserved water or energy, but John Terblanche of Stellenbosch University in South Africa and his colleagues have dispatched those ideas. They collected Cape Lappet moth caterpillars (*Eutricha capensis*) and reared them as individuals and in groups of up to 100. Metabolic rates and water use did not decrease with group size.

Given that aggregating caterpillars did not conserve energy, the researchers suggest that the behaviour may confer other advantages, such as faster growth rates or safety in numbers.

J. Exp. Biol. **http://doi.org/npp (2013)**

ECOLOGY

US forests grow to be different

Trees have made a major comeback in the northeastern United States, but the regrown forest is in some ways markedly different from that of 400 years ago, when European colonists started clearing trees for agriculture.

Jonathan Thompson of the Smithsonian Conservation Biology Institute in Front Royal, Virginia, and his colleagues examined land-survey records from 1620 to 1825 in nine northeastern states. Colonial surveyors often divided up plots of land using 'witness trees' to mark the corners, generally noting the genus, so the authors were able to compare these data with modern forest inventories.

Although most of the same tree species were present in both time periods (with the exception of the now-rare American chestnut, *Castanea dentata*), their relative abundances differ greatly. Colonists removed a forest dominated by species such as beech and hemlock conifers, but today's trees form a canopy of mostly maple and birch.

PLoS ONE **8, e72540 (2013)**

TECHNOLOGY

A cheaper, quieter MRI machine

Magnetic resonance imaging (MRI) is expensive, noisy and requires bulky equipment. It can also have side effects, such as stimulating nerves in patients. These problems arise from the constant switching between positive and negative magnetic-field gradients used to manipulate the spin of hydrogen nuclei throughout the patient's body. The energized nuclei produce radiofrequency signals, which carry the information used to build up an image.

By exploiting the radiofrequency pulses used to prepare the nuclei, Jonathan Sharp at Alberta Innovates Technology Futures in Calgary, Canada, and his

colleagues removed the need for switched magnetic fields. Instead, they manipulated the nuclei using pairs of resonant radiofrequency fields twisted in opposing directions and a static magnetic field. The technique could make MRI cheaper, accessible and quieter.
***NMR Biomed.* http://doi.org/nqf (2013)**

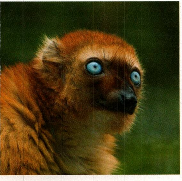
LANGUAGE

Babies hear a primate's call

Babies listen to lemur vocalizations in the same way that they listen to human speech.

A baby's language skills develop rapidly during the first year, and previous research has shown that by three months, hearing human speech while viewing objects helps infants to group objects into categories. Alissa Ferry at the International School for Advanced Studies in Trieste, Italy, and her colleagues examined how recordings of calls from a lemur (*Eulemur macaco flavifrons*; **pictured**) influenced how infants performed when they were asked to discriminate between images of dinosaurs and fish.

The team found that lemur calls helped three- to four-month-old infants to categorize objects but did not help six-month-olds. The study suggests that the link between language and the capacity to categorize objects is initially broad enough to include calls from non-human primates, but quickly becomes tuned to human language.
***Proc. Natl Acad. Sci. USA* http://doi.org/nqx (2013)**



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CELL BIOLOGY

Nuclear receptor linked to fitness

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Would-be dopers may have a new target: the Rev-erb-α protein. Known to regulate sugar and fat metabolism, the nuclear receptor has now been linked to the production and function of mitochondria — the cell's metabolic powerhouses.

A team led by Bart Staels and Hélène Duez, jointly at the Lille II University of Health and Law and the Pasteur Institute in Lille, France, showed that mouse muscle cells lacking Rev-erb-α contain dysfunctional mitochondria, and that mice lacking the gene encoding it, *Nr1d1*, could not run as fast as normal mice. The reverse occurred when the protein was overexpressed or when a synthetic agonist was given, suggesting that Rev-erb-α could be targeted by drugs to improve exercise capacity by boosting mitochondrial number and function in muscle cells.
***Nature Med.* 19, 1039–1046 (2013)**

SYNTHETIC BIOLOGY

Forcing fluorine into molecules

Researchers have discovered a pathway for introducing fluorine atoms into naturally occurring molecules.

Fluorine is present in up to 30% of pharmaceuticals and can expand the usefulness of natural products by, for example, increasing the time they take to break down in the body. But until now, chemists have been using a single method — the fluoroacetate pathway — to insert fluorine into organic molecules.

Michelle Chang of the University of California, Berkeley, and her colleagues have found a different way to insert atoms of the element into a useful group of molecules called polyketides. Their method enlists a soil bacterium (*Streptomyces cattleya*) for the first steps. The bacterium binds fluorine to carbon, making building blocks such as fluoroacetate monomers that can then be inserted into polyketides in the place of acetate.

The team demonstrated the method in the laboratory and in living cells, in which they were able to control where the fluorine atoms ended up in the polyketide molecules.
***Science* 341, 1089–1094 (2013)**

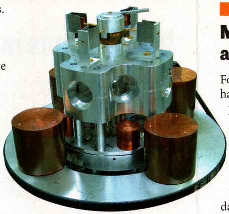
PHYSICS

A startling value for gravitation

The quest to pin down the fundamental constants of nature usually results in increased precision over time, but knowledge of the Newtonian constant of gravitation (*G*), known among physicists as Big G, has not improved much in recent years because different measurement methods continue to disagree.

Now, Terry Quinn and his colleagues at the International Bureau of Weights and Measures in Paris have added to the uncertainty by finding a value for *G* of $6.67545 \times 10^{-11}\ \mathrm{m^3\,kg^{-1}\,s^{-2}}$. This is significantly larger than several other measurements from the past decade, but is in agreement with a 2001 result by the same group using a similar but independent experimental set-up.

The researchers used a torsion balance (**pictured**), in which a thin metal strip changes orientation in response to test masses. The authors say that they do not know why their apparatus gives a different result from other approaches.
***Phys. Rev. Lett.* 111, 101102 (2013)**



TERRY QUINN

PRIMATE COGNITION

Monkeys raise the alarm on predators

For the first time, researchers have shown that non-human primates emit calls in specific sequences to convey the type and location of the threat.

Non-human primates are known to produce calls that signal different kinds of danger. Richard W. Byrne at the University of St Andrews, UK, and his colleagues conducted an experiment on five groups of titi monkeys (*Callicebus nigrifrons*) living in a reserve to find out whether monkey vocalization encoded predator type (raptor or snake), elevation (tree or ground) or both.

A raptor in the air elicited only calls of type A; a raptor on the ground, calls of type A, followed by B. Conversely, a predator on the ground elicited pure B calls, but a ground predator in the trees brought forth B calls, followed by A.
***Biol. Lett.* 9, 20130535 (2013)**

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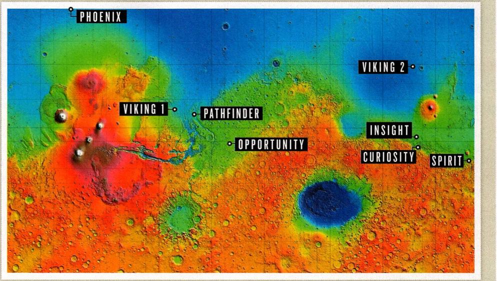
SEVEN DAYS

The news in brief



JPL-CALTECH/NASA

NASA homes in on next Mars landing site

The next US Mars lander will touch down on one of four candidate sites announced by NASA on 4 September. The lander, dubbed Interior Exploration Using Seismic Investigations, Geodesy and Heat Transport (InSight), is set to launch in March 2016 and will study the planet's deep interior to help researchers to understand how rocky planets formed. There were 22 candidates sites; the semi-finalists were chosen for their level and smooth terrain. The sites are all on an equatorial plain in an area called Elysium Planitia, close to where other landers have set up home (pictured). InSight will drill 2–5 metres into the Martian surface, so scientists wanted sites that would not harbour large rocks or solid bedrock that could block the probe.

RESEARCH

Dark-energy survey

A massive sky-mapping project to study dark energy has begun, announced by Fermilab in Batavia, Illinois, on 3 September. The Dark Energy Survey at the Cerro Tololo Inter-American Observatory in Chile launched on 31 August. Over the next five years, it will map 300 million galaxies covering one-eighth of the night sky. Data from its 570-megapixel camera may lead to a better understanding of the mysterious dark energy that is thought to drive the Universe's accelerating expansion. See go.nature.com/ybcueb for more.

Baby-genome plan

Hundreds of babies in the United States will have their genomes sequenced as part of a US$25-million programme unveiled on 4 September by the US National Institute of Child Health and Human Development and the National Human Genome Research Institute. The agencies have chosen four research teams, which will receive grants over the next five years, to test whether sequencing can provide more information than conventional tests that screen newborns for genetic and other disorders. See page 135 and go.nature.com/izz7u9 for more.

High-tech probe

Making better devices for measuring brain activity in animals is the goal of a US$5.5-million collaboration announced on 4 September. Partners include the Howard Hughes Medical Institute in Chevy Chase, Maryland; the Allen Institute for Brain Science in Seattle, Washington; and the nanoelectronics-research company imec in Leuven, Belgium. The project, which also involves University College London and UK biomedical charity the Wellcome Trust, aims to pack more electrodes than ever onto brain probes that record signals between neurons. The group hopes to make the devices available for sale by the end of 2016.

POLICY

Gas phase-down

Leaders of the Group of 20 (G20), representing the world's largest economies, agreed on 6 September to regulate hydrofluorocarbons (HFCs) under the Montreal Protocol on Substances that Deplete the Ozone Layer. Primarily used as refrigerants, HFCs were developed as alternatives to ozone-depleting chemicals, but they are powerful greenhouse gases. Parties to the protocol will debate amending the treaty to implement a global phase-down of HFCs at a meeting in October in Bangkok.

Carbon-tax repeal

The newly elected prime minister of Australia, Tony Abbott, has committed to scrapping the previous government's plans to put a price on carbon emissions. He vowed as part of his campaign to repeal the carbon tax, which outgoing Prime Minister Kevin Rudd had already promised to soften to a carbon-trading scheme. Abbott, who beat Rudd in national elections on 7 September, said that it would be "a very early item of business". But Abbott may face a fight to push the repeal through parliament.

FACILITIES

Spanish clash

Plans to build a particle accelerator and neutron source in Bilbao, Spain, have come to an abrupt halt. The governing board of the €180-million (US$238-million) European Spallation Source-Bilbao (ESS-Bilbao) announced on 29 August that the project's scientific director had been removed, and that the executive director's contract would not be renewed. The board cited a need to analyse and refocus the initiative. ESS-Bilbao, which has encountered budget shortfalls, was launched in 2009 as a test-bed for a larger facility planned for Lund, Sweden.

PEOPLE

Libel suit tossed

On 4 September, the Taipei District Court in Taiwan dismissed a criminal libel suit against environmental engineer Ben-Jei Tsuang of National Chung Hsing University in Taichung. The petrochemical company Formosa Plastics Group (FPG) in Taipei had sued Tsuang for US$1.3 million in damages for suggesting that its emissions were linked to an increased risk of cancer. Tsuang presented his results at a scientific meeting in 2010 and a press conference in 2011, and has since submitted a paper on the topic. A civil lawsuit against Tsuang by FPG is still pending. See go.nature.com/ukmxye for more.

AWARDS

Balzan prizes

Pascale Cossart (**pictured**), a microbiologist at the Pasteur Institute in Paris, has been awarded one of four 750,000-Swiss-franc (US$800,000) Balzan prizes for her work on the molecular biology of pathogenic bacteria and their interaction with host cells. Physicist Alain Aspect of the École Polytechnique in Palaiseau, France, won another of the prizes for his contributions to quantum information processing.

FREDERIC WOIRGARD/LOOK AT SCIENCES/SPL

Lasker award

Richard Scheller of Genentech in South San Francisco, California, and Thomas Südhof of Stanford University in California have won this year's US$250,000 Lasker Award for Basic Medical Research for their work on the molecular mechanisms that underlie the rapid release of neurotransmitters in the brain. Winners of the award, which this year was announced on 9 September, often go on to get a Nobel prize. See go.nature.com/ubuzgy for more.

Ocean contest

The X Prize Foundation is offering US$2 million in a competition, sponsored by US philanthropist Wendy Schmidt, to improve ocean pH monitors, it announced on 9 September. Researchers can compete for one or both of two $1-million prize pots — one for the most accurate and another for the most cost-effective sensor. Subtle changes in ocean acidity can have marked effects on marine life, but existing sensors do not work well at depth or over long periods of time, and are too expensive to deploy widely. See go.nature.com/ttlysw for more.

EVENTS

Lunar orbiter up

On 6 September, NASA launched the Lunar Atmosphere and Dust Environment Explorer (LADEE) from Wallops Flight Facility in Virginia. The orbiter should arrive at the Moon in about 30 days, and will fly 20–50 kilometres above the surface. LADEE will collect dust and gas molecules in the lunar atmosphere, searching for silicon, magnesium and other elements ejected from Moon rocks. Researchers hope that data from LADEE could help to explain the twilight glow above the Moon's horizon seen by missions in the 1960s. See go.nature.com/j2tzmn for more.

Labour row ends

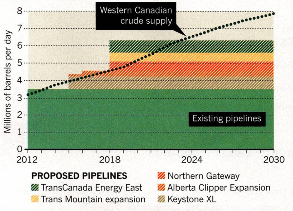
The Atacama Large Millimeter/submillimeter Array (ALMA) observatory restarted operations in Chile on 9 September, after a labour strike shut down the centre for 17 days. Contract negotiations had broken down between the union that represents most of ALMA's Chilean staff and Associated Universities Incorporated, based in Washington DC, which employs the workers (see go.nature.com/vhxfrv). The two-year contract signed on 7 September includes shorter working shifts, a 4% pay rise for union workers at the low end of the wage scale, and a bonus for workers at the highest-elevation ALMA site.

TREND WATCH

With existing oil pipelines at near full capacity, increased production in the oil sands of Alberta, Canada, will depend on new projects such as the controversial Keystone XL, says a report from the Sierra Club in San Francisco, California. The environmental group argues that halting Keystone — expected to carry about 730,000 barrels of oil per day from 2015 — would prevent an increase in greenhouse-gas emissions by impeding further development of carbon-rich oil in the region.

OIL SAND EXPORTS

Companies need new pipelines to sustain production from Canadian oil sands.



SOURCE: NRDC/US DEPT STATE

COMING UP

16–21 SEPTEMBER

The Seventh International Congress on Advanced Electromagnetic Materials in Microwaves and Optics takes place in Bordeaux, France, where scientists will share the latest results in metamaterials research. Topics will include advances in cloaking, and medical applications of metamaterials.

go.nature.com/yqmli6

BUSINESS

Drug resurrected

On 4 September, AstraZeneca launched clinical studies of a previously abandoned experimental ovarian-cancer drug. Researchers had hoped that olaparib would herald a new class of drug targeting a DNA-repair protein called PARP, but the London-based pharmaceutical firm halted the drug's development in 2011 after a failed clinical trial (see *Nature* **483,** 519; 2012). Further analysis suggested that olaparib may work best against cancers bearing mutations that affect other DNA-repair proteins, leading the company to launch the latest trial in patients with *BRCA*-mutant cancers.

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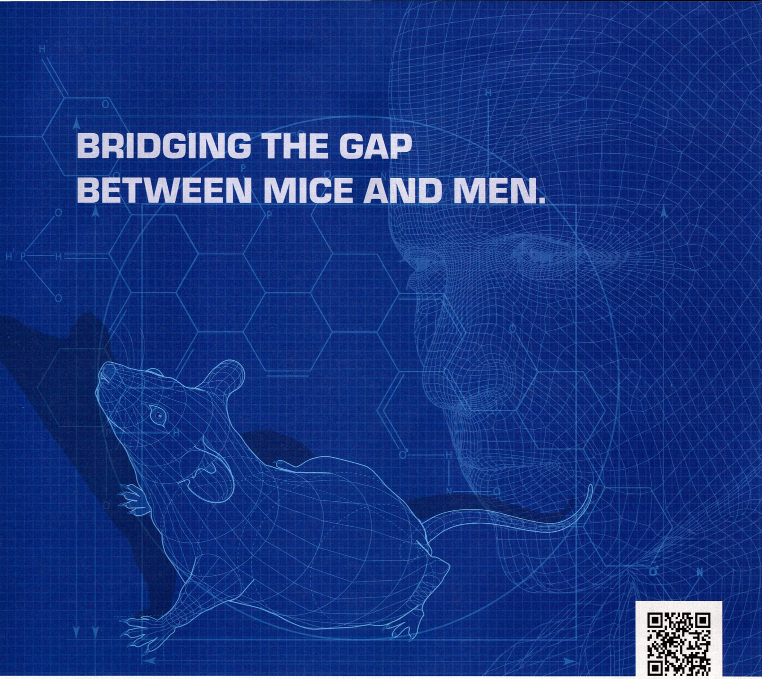

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NEWS IN FOCUS

UK POLICY Plan to offload labs vexes environmental scientists **p.145**

ENERGY Chemical databases pave the way for 'greener' fracking fluids **p.146**

FUNDING More cuts loom for US science as sequestration drags on ... and on **p.147**

TAXONOMY On the trail of the spy who chased lizards and frogs **p.150**

LYNN M. STONE/NATURE PICTURE LIBRARY

The grey wolf, protected for more than 30 years, could see its endangered status removed nationwide.

CONSERVATION

Grey wolves left out in the cold

US plan to remove federal protection elicits howls of protest.

BY CHRIS WOOLSTON

Central Kentucky is coyote country. But the 33-kilogram animal shot by a hunter near Munfordville this spring was definitely not a coyote. Its huge paws, broad snout and massive build suggested that it was a grey wolf (*Canis lupus*) — the first to be shot in Kentucky in more than 150 years. DNA tests confirmed the animal's identity in August.

The animal, a possible stray from hundreds of kilometres away in Michigan or Minnesota (although it cannot be ruled out that it was once captive), was also a player in a growing debate that mixes science, politics and passionate public opinion. From Kentucky to California, wolves are forcing biologists and policy-makers to re-examine the US Endangered Species Act (ESA) and the very definition of an 'endangered' species.

The act, introduced in 1973, was a landmark piece of legislation. Its purpose has been contentious ever since, but it is intended to save species "in danger of extinction throughout all or a significant portion" of their range. Although wolves have never been at risk of extinction in the United States as a whole, those in the 48 contiguous states were classified as endangered in 1978.

➲ **NATURE.COM**
Why the US wildlife service wants to list a new wolf species:
go.nature.com/7nujx9

After decades of federal protection and reintroduction programmes, the US Fish and Wildlife Service (FWS) undertook a comprehensive review, which found that wolf populations near the western Great Lakes and the northern Rocky Mountains had recovered sufficiently to warrant removing ESA protection (see 'Wolf pack'). (There are now about 4,000 wolves in the Great Lakes area and nearly 1,700 in the northern Rockies.) Wolves in these areas were 'delisted' between May 2011 and August 2012.

But in June this year, the FWS proposed removing ESA protection from all US grey wolves, citing the earlier review as evidence of their recovery and arguing that the original listing had erroneously included regions outside the species' historical range. The agency says that by delisting the rest of the US wolf population, it can concentrate its resources on ESA protection for the Mexican wolf (*Canis lupus baileyi*), a subspecies of the grey wolf.

The proposal marks a turning point for the grey wolf. A century ago, the animals had been hunted almost out of existence south of the United States–Canada border. Now, as a result of the partial delisting, six states have wolf-hunting seasons. In Montana, 225 wolves were legally trapped or shot in the 2012–13 season.

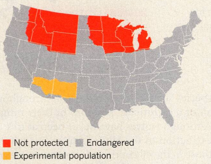
Grey wolves removed from the ESA programme would be managed by states, some of which have in the past shown little interest in protecting wolves or expanding their territory. Delisting the wolves would essentially prevent them from reclaiming large parts of their historic range in places such as California, the southern Rockies and the northeast, says John Vucetich, a forest scientist at Michigan Technological University in Houghton. And, as the appearance of the wolf in Kentucky suggests, pushing boundaries is a wolf speciality.

"The Fish and Wildlife Service is essentially saying that this is the best that wolves can do, and it's not even close," he says. "Wolves are on the verge of setting a precedent for the Endangered Species Act."

Robert Wayne, an ecologist and evolutionary biologist at the University of California, Los Angeles, says that wolves need broad ranges and large populations to return to their historic ▶

WOLF PACK

By October 2012, grey wolf populations in six US states had recovered such that they no longer needed protecting by the Endangered Species Act.



SOURCE: US FISH AND WILDLIFE SERVICE

▶ levels of gene flow and diversity. In 2005, he and his colleagues analysed mitochondrial DNA from specimens collected before wolves were decimated in the 1900s, and found that it contained twice as many variations as DNA from modern wolves (J. A. Leonard *et al. Mol. Ecol.* **14,** 9–17; 2005). The researchers estimated that the wolf populations in Mexico and the western United States had once reached 380,000 individuals. "Wolves have not recovered over a large part of their range," Wayne says.

But Gary Frazer, assistant director for endangered species at the FWS in Arlington, Virginia, says that the service exceeded its own minimum targets for wolf recovery as early as 2001, and thus it is a case of mission accomplished. "That was the plan from the beginning: to declare recovery, to delist the species, and to move on to other species that need our attention," he says, noting that the agency's resources are limited.

Wolves might occupy only a fraction of their historic range, but they are not in danger of extinction, adds Mark Boyce, a biologist at the University of Alberta in Edmonton, Canada. "We have 6,000 wolves in Alberta alone," he says. "Except for Mexican wolves, the populations in the lower 48 states add nothing to the genetic diversity of the species." Boyce believes that any expansion of the wolves' range would be costly for ranchers. In 2011, he co-authored a study that tracked wolves using the Global Positioning System, showing that each wolf pack in southwestern Alberta killed an average of 17 cattle every year (A. T. Morehouse and M. S. Boyce *Front. Ecol. Environ.* **9,** 440–445; 2011).

The wolf controversy highlights the strained relationship between science and politics. Vucetich and Wayne, along with Roland Kays of the North Carolina Museum of Natural Sciences in Raleigh, were, they claim, dropped in August from a panel to review the FWS proposal because they had publicly opposed the wolf's delisting. "I'm not mad about not being on the panel, but it doesn't seem like they were following proper procedure," Wayne says. "It was punitive," he claims.

The review process has since been restarted. "We still haven't figured out how to handle a situation where experts have outspoken views," Frazer says. "We are not an academic institution. We're trying to implement federal law." The public consultation period will close in October, but because the panel's peer review will not be complete by then, Frazer plans to reopen public comments in January 2014. "People are very passionate about wolves," he says. The final decision may take a year or more.

The future of US wolves will hinge mainly on public acceptance of their delisting. Groups such as Defenders of Wildlife in Washington DC protest against wolf hunting, whereas those affiliated with hunters and ranchers want wolves to be aggressively controlled. Some individuals have made death threats to ranchers who legally shot wolves that attacked livestock.

Vucetich thinks that the government is eager to pass the issue on to the states. "It saps the energy of people working on it," he says. ■

MATHEMATICS

Physicists net fractal butterfly

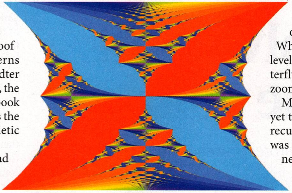
Decades-old search closes in on recursive pattern that describes electron behaviour.

BY DEVIN POWELL

After a nearly 40-year chase, physicists have found experimental proof for one of the first fractal patterns known to quantum physics: the Hofstadter butterfly. Named after Douglas Hofstadter, the Pulitzer prizewinning author of the 1979 book *Gödel, Escher, Bach*, the pattern describes the behaviour of electrons in extreme magnetic fields.

To catch the butterfly, scientists have had to fashion innovative nets. Since May, several groups have published experiments that sought the pattern using hexagonal lattices of atoms; last month, others reported seeking it with atomic laser traps. Some physicists say that studying the pattern could help in the development of materials with exotic electric properties. But the main point of the chase was to check whether the butterfly looks as predicted.

"Hofstadter's concept was initially disturbing to a lot of people," says Cory Dean, an experimental physicist at the City College of New York. "Now we can say his proposal wasn't so crazy after all."



Hofstadter's butterfly describes electron motion.

DOUGLAS HOFSTADTER

Hofstadter, now a cognitive scientist at Indiana University Bloomington, sketched out the pattern in the 1970s while a graduate student in physics. It was known at the time that electrons under the influence of a magnetic field would race around in circles. But Hofstadter found that in theory, if the electrons were confined inside a crystalline atomic lattice, their motion would become complicated. As the magnetic field was cranked up, the energy levels that define the motion of electrons would split again and again. When represented on a graph, those energy levels revealed a pattern that looked like a butterfly — and continued to do so, even when zoomed in to infinitely small scales.

Mathematician Benoit Mandelbrot had yet to popularize the term 'fractal' for such recursive patterns, and Hofstadter's adviser was unimpressed. "He scornfully called the nesting pattern that this upstart youngster claimed to see, 'mere numerology'," says Hofstadter. "He even told me that I would be unable to get a PhD for this kind of work." Hofstadter published[1] his description of the butterfly in 1976, after finishing his PhD.

The idea was difficult to test. The strength of the required magnetic field depends on the spacing between the atoms in the lattice. In conventional materials, in which atoms are separated by less than one-billionth of a metre, the pattern can emerge only in fields on the order of tens of thousands of tesla. The best available magnets can reach only about

100 tesla, and for just a fraction of a second.

But smaller fields are sufficient in lattices with larger spacings, which can be created by layering materials in stacks. In May, researchers reported[2] that they had stacked a single sheet of graphene, in which carbon atoms are arranged like a honeycomb, on top of a sheet of honeycombed boron nitride. The layers create a repeating pattern that provides a larger target for magnetic fields than the hexagons in each material — effectively magnifying the field.

After applying a field, the researchers measured discrete changes in the conductivity of the composite material — stepwise jumps that result from splits in the energy levels of its electrons. These were not a direct detection of the expected electron behaviour, but were a proxy for it. Hofstadter's butterfly had not quite flown into the net, but it had revealed its existence. "We found a cocoon," says Pablo Jarillo-Herrero, an experimental physicist at the Massachusetts Institute of Technology (MIT) in Cambridge. "No one doubts that there's a butterfly inside."

Nobel laureate Wolfgang Ketterle, another physicist at MIT, is going after the butterfly in a different way: by making atoms act like electrons. To do this, he chills rubidium atoms to a few billionths of a degree above absolute zero, and uses lasers to trap them in a lattice with egg-carton-like pockets.

When zapped by an extra pair of criss-crossed lasers, the atoms tunnel from one pocket to another. Tilting the grid allows gravity to guide the atoms into paths that mimic the circular motions of an electron in a magnetic field — although no actual magnetic fields are involved. The system can easily track the motion of individual atoms, and should be able to mimic a magnetic field strong enough to produce a Hofstadter's butterfly. "Cold atoms will give us an enormous freedom," says Ketterle, whose team posted its study on the preprint server arXiv last month[3]. But the set-up has a liability: the lasers tend to heat the cold atoms, limiting the ability to control the energies of the particles and reveal the fractal pattern.

Still, if the heat can be handled and the butterfly simulated, this system could be a starting point for exploring quantum behaviours in solids, such as materials that can conduct electricity on the surface but are insulators at the core. Dieter Jaksch, a physicist at the University of Oxford, UK, says, "I expect that a wealth of new phenomena and insights will be found when exploring the butterfly." ■

1. Hofstadter, D. R. *Phys. Rev. B* **14,** 2239–2249 (1976).
2. Hunt, B. *et al. Science* **340,** 1427–1430 (2013).
3. Miyake, H., Siviloglou, G. A., Kennedy, C. J., Burton, W. C. & Ketterle, W. Preprint at http://arxiv.org/abs/1308.1431 (2013).

ENVIRONMENTAL SCIENCES

Hackles rise over privatization plan

UK Natural Environment Research Council proposes to cut four institutes loose, but scientists fear for long-term data.

BY DANIEL CRESSEY

The UK Natural Environment Research Council (NERC) is in a quandary. The government body, which channels money to environmental scientists, has for weeks been soliciting evidence on whether it should hand funding control of four of its five key research institutes to the private sector. The move is meant in part to decrease the institutes' reliance on waning government funds, but leading scientists have now gone public with their concerns that it could jeopardize research and data of crucial importance to environmental science in the United Kingdom and around the world.

At stake are the futures of the National Oceanography Centre, the British Geological Survey, the Centre for Ecology and Hydrology and the National Centre for Atmospheric Science. (The British Antarctic Survey, which NERC also runs, is not affected.) As well as conducting research on a variety of environmental topics, all four are closely linked to specialist centres that collect long-term data, such as the British Oceanographic Data Centre, hosted by the National Oceanography Centre in Liverpool. In total, the institutes have a budget of about £400 million (US$628 million).

"We need safeguards for these unique assets and long-term, large-scale perspectives."

"The NERC centres uniquely provide long-term consistent data, and make them freely available for the benefit of ecological science and to improve our understanding of the natural world," says William Sutherland, president of the British Ecological Society in London. "These data include studies that are undertaken over the course of decades, protected from changes in fashion or the fluctuations of short-term demands. Any change in ownership of the centres must preserve this."

Helen Snaith, a remote-sensing researcher at the National Oceanography Centre in Southampton and a trade-union representative, notes that advice that the centres provide to the government could be compromised if they start generating significant income from private sources. "There's the potential for a very clear perceived conflict of interest," she says. She also worries that the roughly 1,750 members of staff at the four centres, about two-thirds of whom are researchers, could get a worse deal on pay and benefits under private ownership.

Duncan Wingham, NERC's chief executive, stresses that no decision has yet been taken. If the centres are moved out of the public sector, he says, it would not necessarily mean that they become profit-making. They could, for example, become part of universities. He has also emphasized that the decision on the centres' futures will not consider cost savings, which most interested parties concede.

There may also be advantages, adds Wingham — notably that freeing the institutes of public-sector constraints on pay and promotion, and from reliance on government funding, could give them better flexibility to respond to new opportunities.

Steve Ormerod, an ecologist at Cardiff University and chairman of the Royal Society for the Protection of Birds, acknowledges this. He sees advantages if the Centre for Ecology and Hydrology can develop partnerships on its own terms with international agencies and businesses, and says that being independent might allow the centre to win more funding and attract more researchers.

But there are risks, he says. "We need safeguards for these unique assets, skills and long-term, large-scale perspectives that have always provided crucial support for impartial, highly rigorous, evidence-based advice."

NERC's call for evidence on the proposal closed at the end of August, and submissions are being reviewed. The NERC board will decide on the institutes' futures in December. If the research council does choose to divest itself of these centres, the decision would represent almost the end of an era for government-controlled science in the United Kingdom. According to its 2011–12 report, the UK Biotechnology and Biological Sciences Research Council has made arrangements to "remove [its] ability to exert control" over some of its institutes; and the Medical Research Council is transferring some of its in-house units to universities. ■

ENVIRONMENTAL CHEMISTRY

Secrets of fracking fluids pave way for cleaner recipe

Disclosure of chemicals used in hydraulic fracturing will empower green chemistry.

BY JEFF TOLLEFSON

The myriad liquid concoctions used in hydraulic fracturing make for quite a recipe book. Since January 2011, FracFocus, an online chemical-disclosure registry, has assembled a list of the mixtures used at more than 52,000 oil and gas wells across the United States. In these data, geochemist Brian Ellis sees opportunity. He plans to mix different chemicals into oil- and gas-rich shale rock inside a pair of high-pressure chambers that he is building. This will allow him to explore the reactions that occur when these 'fracking' fluids are injected deep underground.

The fluids, which are mixed with sand, are predominantly water, laced with 1% 'special sauce'. The recipes for that fraction — a mixture that includes acids, solvents and corrosion inhibitors — were until the last few years secrets guarded by the companies that seek to penetrate shale formations to release stores of fossil fuels. But in the face of widespread concern about water contamination, 21 US states have adopted mandatory disclosure rules for the mixtures, making it easier for scientists such as Ellis, of the University of Michigan at Ann Arbor, to assess their impacts.

The chemicals used in hydraulic fracturing to extract oil and gas are being disclosed to online registries.

DAVID ZALUBOWSKI/AP/PRESS ASSOC. IMAGES

Much of the data end up in registries such as FracFocus, which is overseen by state energy and water organizations (see 'A recipe for fracking'). "There are still a lot of bugs, but the vast majority of companies are now disclosing their chemicals," says Scott Anderson, a senior policy adviser for the Environmental Defense Fund in Austin, Texas, which advocates for greener fracking procedures.

More than 500 companies have reported data to FracFocus so far. Academic researchers, advocacy groups and companies are now poring over those recipes to assess their toxicity in the hope of narrowing them down to a group of environmentally acceptable ones — and perhaps spurring the synthesis of even greener alternatives.

The boom in the disclosure of fracking-fluid components has occurred despite the fact that the federal government has yet to weigh in with its own rules. The Department of the Interior has proposed requiring the disclosure of chemicals used during hydraulic-fracturing operations on public lands, but much of the current oil and gas development is taking place on private land. Many companies are volunteering the information anyway, even in states that have no disclosure requirements. And companies that do the hydraulic fracturing, such as Halliburton and Baker Hughes, both based in Houston, Texas, are developing their own chemical-assessment programmes in an apparent effort to address public concerns and reduce their environmental footprint.

The data in these registries, although increasingly abundant, remain incomplete, unconsolidated and difficult to compare. The European Union is phasing in a unified chemical-regulation programme that governs reporting across all commercial sectors. Energy companies operating in the North Sea, for example, must all play by the same rules and abide by strict reporting requirements. But in the United States, the regulations on chemical reporting remain a mixture of state and national policies that vary by industry.

➲ NATURE.COM
For concerns about the impact of fracking, see:
go.nature.com/l93vas

Even in states with disclosure laws, companies can omit information in the interests of protecting intellectual property. For example, a subsidiary of ExxonMobil, based in Irving, Texas, declined to list the components of a gelling agent — used to help to suspend sand in water — at one of its wells in Wyoming, calling the information a "trade secret".

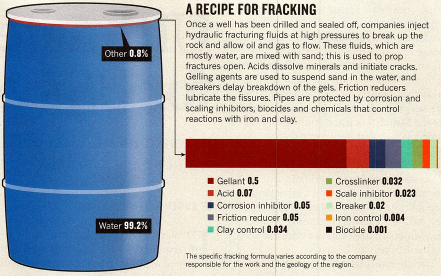
The result is that companies are still operating under their own risk assessments and not disclosing all of the information that might be needed for independent verification. "If everybody has a different definition of what is hazardous and doesn't fully disclose the chemicals they use, then it is going to be awfully difficult to compare," says Lauren Heine, co-director of Clean Production Action, an advocacy group based in Somerville, Massachusetts.

Heine's group is sifting through company disclosures to perform a risk assessment on the most commonly used chemicals. The effort is designed to provide a single point of comparison so that scientists, industry and the public can make informed decisions about which chemicals are best.

Daniel Durham, who heads a chemical-assessment programme at the Houston-based energy company Apache, says that although Heine's effort is promising, companies do not need to wait. The US Environmental Protection Agency (EPA) already maintains its own public registry of preferred chemicals for various industrial processes. Companies that want to register their chemicals provide the EPA with toxicity and environmental-assessment data; the registry also allows companies to keep certain data confidential if intellectual property is involved.

The upshot is a growing — albeit incomplete — list of preferred chemicals that companies such as Apache can choose from as they design their fracking fluids. A company that wants to avoid using a solvent such as ethylene glycol monobutyl ether, for example — used to reduce viscosity but possibly toxic to the endocrine system — could look through the EPA list for alternatives. "It's a very good road map to green chemistry," Durham says.

Eventually, Durham hopes that researchers will help to develop novel chemicals that could be used to make the entire hydraulic-fracturing process cleaner and more efficient. Scientists such as Ellis could play an important part.



A RECIPE FOR FRACKING

Once a well has been drilled and sealed off, companies inject hydraulic fracturing fluids at high pressures to break up the rock and allow oil and gas to flow. These fluids, which are mostly water, are mixed with sand; this is used to prop fractures open. Acids dissolve minerals and initiate cracks. Gelling agents are used to suspend sand in the water, and breakers delay breakdown of the gels. Friction reducers lubricate the fissures. Pipes are protected by corrosion and scaling inhibitors, biocides and chemicals that control reactions with iron and clay.

The specific fracking formula varies according to the company responsible for the work and the geology of the region.

Ellis wants to know whether fracking fluids are contributing to geochemical reactions within the shale rock that might free up potentially dangerous metals and radionuclides, such as arsenic, barium, strontium and uranium. These elements are often found in trace concentrations in the waste water produced by oil and gas companies, but can also be found naturally in groundwater. Ellis eventually hopes to help companies to select better chemicals that would minimize the potential for contamination and the need for waste-water treatment. But for now, he says, he is focused on the basic science. "Fundamentally, I just want to understand those reactions a little better." ■

POLICY

More cuts loom for US science

Stalemate in Congress puts spending plans on hold.

BY LAUREN MORELLO

Laura Niedernhofer is counting her pennies. The mid-career molecular biologist moved last year to the Scripps Research Institute's campus in Jupiter, Florida — a risky decision that saw her building a new laboratory group at a time when the US government was cutting its support for science. In June, Niedernhofer abandoned one of her main lines of research — reducing the toxicity of cancer drugs — after the National Institutes of Health (NIH) rejected her grant application. In July, the agency approved a second grant, allowing her to keep another research thrust alive — on the molecular mechanisms of ageing. But the NIH cut the award by 18%, preventing her from hiring an additional postdoctoral researcher.

Niedernhofer is not alone. In a survey of more than 3,700 US scientists released on 29 August by the American Society for Biochemistry and Molecular Biology in Rockville, Maryland, one-third said that they had laid off researchers, and close to two-thirds had seen their funding fall since 2010. Federal spending on research and development has declined by 16.3% since 2010, the fastest drop in a three-year period since the end of the space race in the 1970s, according to an analysis published on 3 September by the American Association for the Advancement of Science in Washington DC.

The most drastic reduction occurred on 1 March, when across-the-board budget cuts known as sequestration lopped 5% from the budgets of most government agencies. Science powerhouses such as the NIH in Bethesda, Maryland, and the National Science Foundation in Arlington, Virginia, began to scrimp by reducing the values and durations of grants, and the number of recipients per application cycle.

> *"There is continuing pressure for additional budget cuts as a price for raising the debt ceiling."*

The situation could worsen in the coming months. Congress, which returned to Washington DC this week, has made little progress on setting government spending for the 2014 fiscal year, which begins on 1 October. An attempt by a group of Republican senators and the White House to negotiate an agreement on deficit reduction broke down in late August, and since then the crisis in Syria has diverted the attention of Congress. To avoid a government shutdown, lawmakers are expected to extend current funding levels until December. That extension, known as a continuing resolution, would run out at about the same time that the country confronts another financial matter: surpassing its borrowing limit, or debt ceiling.

That could set up a budget battle royal in the next few months. A similar fight in the summer of 2011 led to the law that created sequestration; it specifies annual spending reductions until 2021, if Congress does nothing to override it. The next round of cuts, scheduled to take effect in January 2014, would trim spending to 2% below the already-whittled-down 2013 level.

Indications of how the various science agencies will fare can be found in Congress's ▶

▶ unfinished spending bills. The Democratic-controlled Senate would eliminate sequestration and give science agencies modest boosts. The Republican-controlled House of Representatives would cut funding in many areas to keep total spending in line with the 2% cut that sequestration prescribes. One relatively bright spot is the House's proposed allocation of $7.0 billion for the National Science Foundation, the same amount that the agency received in 2012 — a bountiful level in House terms. But Barry Toiv, vice-president for public affairs at the Association of American Universities in Washington DC, worries that House Republicans will now end up seeking cuts beyond 2%. "There is continuing pressure for additional budget cuts as a price for raising the debt ceiling," he says.

The situation leaves US research institutions in an uneasy position, unsure whether 2013 funding levels will have been the nadir, or a prelude to something worse. Many are just beginning to feel the effects, because of the delay between the sequestration cuts and grants being awarded. At the University of Maryland in College Park, the haul of grants from the NIH was 7% below projections in the 2012–13 academic year, and its share of defence-department cash was 3% lower than expected, says chief research officer Patrick O'Shea. At Johns Hopkins University in Baltimore, Maryland, four doctoral programmes in the department for environmental health sciences each accepted just one student this year, instead of the usual two or three, says Jonathan Links, a medical physicist who handles the university's crisis planning.

But US universities collect tuition fees and generally have endowments, which means that they have ways to provide stopgap funding to scientists in tight spots. At a 'soft-money' research institute such as Scripps, grants are needed to pay almost all the bills — so Niedernhofer's situation was more dire.

Although she is continuing her ageing research with her three postdocs, she has a new standard question she asks before hiring them: will they consider only academic research as a job? "I can't guarantee that they will get that," she says, "and I don't want to be the one to break their hearts." ■

PHYSICS

Ultimate upgrade for US synchrotron

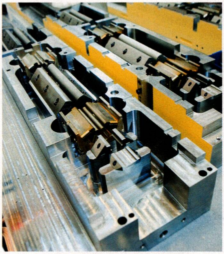
Argonne lab banks on beam-bending magnets in bid for world's most focused X-ray light source.

BY EUGENIE SAMUEL REICH

Every day, in dozens of synchrotrons around the globe, electrons are whipped around in circular storage rings to provoke them into emitting X-rays, useful for imaging materials, identifying chemical-reaction products and determining crystal structures.

But photon scientists do not want just any old storage ring. For more than a decade, they have dreamt of 'ultimate' storage rings — ones that use specialized magnets to produce X-ray beams that are as tightly focused as theory allows.

Now, researchers at the largest US synchrotron, the Advanced Photon Source (APS) at the Argonne National Laboratory in Illinois, are taking steps to develop this technology. In the process, they hope to leapfrog several international facilities that have a head start.


DANFYSIK

Magnets for the Swedish MAX IV synchrotron.

In Sweden, ultimate-storage-ring technology is being pioneered at MAX IV, a 528-metre-circumference synchrotron in Lund. Scientists there first sought to increase the intensity and brightness of the synchrotron's X-ray light in 2006 by focusing electron beams more tightly. The design relied on groups of seven magnets, known as multi-bend achromats, that could be used in as many as 20 places around the ring to nudge the paths of electrons back and forth until they lined up more-or-less perfectly. Machine director Mikael Eriksson recalls that when he toured US light sources to describe the project, "few believed it".

Eriksson now has believers. In a report posted online on 29 August, researchers at the Argonne lab describe how they are hoping to upgrade the 1.1-kilometre-circumference APS with multi-bend achromats (see go.nature.com/asxrqb). "There's a new technology that has come along and it's pretty revolutionary," says APS director Brian Stephenson. Current storage rings have at most double-bend achromats, which contain two magnets rather than seven. Physicists had thought that including more magnets would make the beam unstable by bending it too much and introducing too many fluctuations. But the work at MAX IV showed that very compact magnets enable bending paths that are short enough to stop fluctuations from building up.

The US Department of Energy, which funds the APS, still needs to approve the plan. In July, one of the department's advisory committees suggested that US labs were being left behind while other countries push towards ultimate storage rings. The committee had also recommended pursuing a next-generation X-ray

MORE ONLINE

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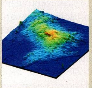
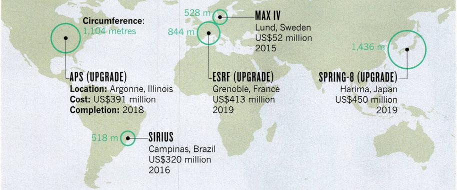
IMAGE OF THE WEEK


WILLIAM SAGER/UNIV. HOUSTON

Volcano found under Pacific Ocean is Earth's largest at 650 kilometres wide **go.nature.com/beeqp4**

FOCUSED BEAMS

Five synchrotron facilities are developing special magnets so that they can become ultimate storage rings.



APS, Advanced Photon Source; ESRF, European Synchrotron Radiation Facility.

SOURCE: US DEPARTMENT OF ENERGY/BESAC; MAX IV

laser, useful for making 'molecular movies' of chemical reactions, among other things (see *Nature* **500**, 13–14; 2013). But such a laser would have limitations: its strongly peaked light pulses would destroy delicate materials. Ultimate storage rings, by contrast, satisfy a need for more gradually peaked pulses of light.

Researchers say that these storage rings could revolutionize X-ray imaging by making it possible to map evolving chemical processes. Current X-ray sources are not bright enough to track changes in materials with nanometre and nanosecond resolution, because there are not enough coordinated photons in the beams. Ultimate storage rings would change that. "A whole class of new problems opens up," says Paul Evans, a materials scientist at the University of Wisconsin–Madison. For example, he says that the rings could be used to investigate what happens chemically and electrically at the interface between materials inside a battery as it runs out.

The APS is seeking to tack the installation of ultimate-storage-ring technology on to a separate upgrade that had already been approved. Cost calculations are still ongoing, but Stephenson hopes that the multi-bend achromats can be included without raising the upgrade budget much above US$391 million. MAX IV is implementing the technology for only 340 million Swedish kronor ($52 million), but that ring is smaller and the price tag would not include the overhead costs that are charged at US energy-department labs.

After its upgrade, the APS could surpass MAX IV by approaching the theoretical limit for the most focused beam possible. The Swedish synchrotron will contain 20 multi-bend achromats, whereas the APS upgrade calls for around 40. In 2012, physicists at SLAC National Accelerator Laboratory in Menlo Park, California, showed that the number of multi-bend achromats around a larger ring could be pushed even higher without fundamentally destabilizing the electron beam. "The key is to make the bending gentle," says Yunhai Cai, head of beam physics at SLAC.

Alongside APS, the European Synchrotron Radiation Facility (ESRF) in Grenoble, France, has also opted for a multi-bend-achromat upgrade, after a working group concluded last October that the technology was affordable. ESRF director-general Francesco Sette says that accelerator physicists there showed that multi-bend achromats could work with the facility's existing injector, a part of the machine that supplies extra electrons to the main ring a few times each day. He had previously thought that a new injector would be needed. "We are today in full swing to launch as soon as possible," he says.

Storage rings in Brazil and Japan will also be upgraded with multi-bend achromats, giving MAX IV a window of only one year from its projected completion date of 2015 before it faces competition (see 'Focused beams').

Some have suggested that particle-physics tunnels, too, could eventually be turned into light sources with multi-bend achromats. SLAC has an idle 2.2-kilometre-circumference tunnel that originally housed a particle accelerator used to compare the decay rates of matter and antimatter. And a 6.3-kilometre tunnel used by the now-closed Tevatron particle accelerator at Fermilab near Batavia, Illinois, is another candidate for conversion. Eriksson says that building ultimate storage rings of that size would not be realistic for Sweden, given the relative size of its science budget.

He knows that Sweden's time in the vanguard will be short-lived, and has mixed feelings about seeing other countries adopting the technology that he and his colleagues pioneered so enthusiastically. "We are both happy and a little sorry," he says. ■

CORRECTION

The News story 'NASA ponders Kepler's future' (*Nature* **501,** 16–17; 2013) inflated the size of asteroids that the probe could watch for — they would be several hundred metres in diameter rather than several hundred kilometres.

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Edward Taylor, spy and herpetologist, in 1912.

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THE SPY WHO LOVED FROGS

To track the fate of threatened species, a young scientist must follow the jungle path of a herpetologist who led a secret double life.

BY BRENDAN BORRELL

Before leaving for the Philippines as an undergraduate in 1992, Rafe Brown scoured his supervisor's bookshelf to learn as much as he could about the creatures he might encounter. He flipped through a photocopy of a 1922 monograph by the prolific herpetologist Edward Taylor, and became mesmerized by a particular lizard, *Ptychozoon intermedium*, the Philippine parachute gecko. With marbled skin, webs between its toes and aerodynamic flaps along its body that allow it to glide down from the treetops, it was just about the strangest animal that Brown had ever seen.

Brown learned that Taylor had collected the first known example, or type specimen, near the town of Bunawan in 1912, and had deposited it at the Philippine Bureau of Science in Manila. But the specimen had been destroyed along with the building during the Second World War, and the species had never been documented again in that part of the country. "What are the chances I'm going to see one of the rarest geckos in the world?" he wondered.

He was driven by more than curiosity. Given the rampant deforestation in that part of the Philippines, he wanted to determine whether the species still existed there and if so, how similar it was to geckos collected in other areas. He wanted to see, in other words, whether Taylor's 70-year-old taxonomic decisions were still valid.

On their first night in the field, Brown and his colleagues drove to the edge of the forest and caught two red eyes in the beam of a headlamp. It was a *Ptychozoon*. Back at their hotel, Brown photographed the gecko, took tissue samples for DNA sequencing, and carefully prepped it and stuck it in a jar. It became the neotype to replace Taylor's lost specimen, and in 1997, Brown published a new description of the species[1]. It marked the start of an obsession.

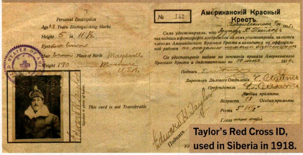
As Brown made his career studying biodiversity in the Philippines over the next two decades, he could not escape Taylor's long shadow. The elder herpetologist had logged 23 years in the field over his lifetime, collecting more than 75,000 specimens around the world, and naming hundreds of new species.

There is a darker side to Taylor's legacy, however. He was a racist curmudgeon beset by paranoia — possibly a result of his mysterious

CLOCKWISE FROM TOP LEFT: SPENCER RESEARCH LIBRARY/UNIV. KANSAS LIBRARIES; COURTESY JOHN TEWELL/PHOTOGRAPHER UNKNOWN; US NATL ARCHIVES; RAFE BROWN; SPENCER RESEARCH LIBRARY/UNIV. KANSAS LIBRARIES

Edward Taylor in the field.

The Philippine Bureau of Science in Manila was destroyed in the Second World War.


Personal Description
Age 28 Years Distinguishing Marks
Height 5 in. 11½
Eyes dark brown
Hair brown Place of Birth Maysville, Missouri, U.S.A.
Weight 190
Signature & photo of bearer.
This card is not Transferable

№ 142

Американскій Красный Крестъ.

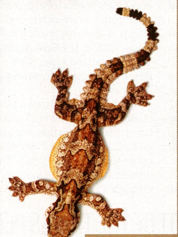
Владивостокъ 9/IX 1918 г.

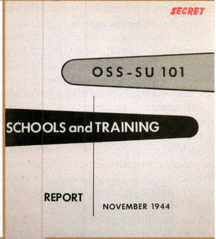
Симъ удостовѣряемъ, что Эдуардъ Х. Тейлоръ чья подпись и фотографія изображены на семъ удостовѣреніи, является членомъ Американскаго Краснаго Креста и находится на оффиціальной работѣ по оказанію помощи бѣженцамъ

Сіе удостовѣреніе выдано на основаніи приказа Американскаго Краснаго Креста и дѣйствительно по 30 іюня 1919 г.

Подпись
Директоръ Дѣлового Отдѣленія
Предсѣдатель
Личныя примѣты.
Возрастъ 28 Особыя примѣты.
Ростъ 5' 11½"
Глаза темно-карие
Подпись Edward H. Taylor

Taylor's Red Cross ID, used in Siberia in 1918.


The Philippine parachute gecko.


SECRET

OSS-SU 101

SCHOOLS and TRAINING

REPORT

NOVEMBER 1944

A report on the Office of Strategic Services jungle-training programmes.

double life as a spy for the US government. He had amassed no shortage of enemies by the time he died in 1978. An obituary noted that he was, to many, "a veritable ogre—and woe to anyone who incurred his wrath"[2]. More damaging, perhaps, were the attacks on his scientific reputation. After the loss of his collection in the Philippines, many of the species he had named were declared invalid or duplicates. The standards of taxonomy had advanced beyond Taylor's quaint descriptions, and without the specimens to refer to, his evidence seemed flimsy.

Nevertheless, Brown felt a connection with his maligned predecessor. It was a bond that intensified when, in 2005, Brown became curator of herpetology at the University of Kansas Natural History Museum in Lawrence, the same institution at which Taylor had spent much of his career. Over the years, Brown has rebuilt some of Taylor's collection and resurrected many of his species. Now, as he finishes a major monograph on a group of Philippine frogs, he is more convinced than ever: "Taylor was right."

Brown's reassessment could prove crucial. Since Taylor's time, taxonomy has become more than just a naming exercise. Designating a group of organisms as a new species, or lumping it in with an old one, can affect the animals' legal protection and influence the allocation of scarce conservation resources. Amphibian declines, in particular, have made headlines around the world, and the Philippines ranks second only to Sri Lanka for sheer proportions of imperilled species: 79% of Philippine amphibians are found nowhere else on Earth, and 46% are under threat of extinction. But following Taylor's trail has given Brown cause for optimism. "A lot of the things people thought were extinct," he says, "if you go right where Taylor said to go, you can find them."

"HE WAS A VERITABLE OGRE — AND WOE TO ANYONE WHO INCURRED HIS WRATH."

A LUST FOR ADVENTURE

On the fourth floor of the Kansas museum, Brown is walking through the herpetological collections. Lizards float upside down in yellow-tinged alcohol. Snakes coil like corkscrews, and two dozen tiny, dark frogs embrace in a specimen jar. On one shelf, the jars have red ribbons tied around their lids to signify that their contents are type specimens: the standards on which species descriptions are based.

When scientists disagree on whether something is a new species or a variant of a known one, they often need to refer back to the type specimen or even return to where it was collected. Brown opens a jar and extracts a small lizard that has a tin tag tied to its waist with twine. It is one of Taylor's originals, on loan from the California Academy of Sciences in San Francisco. "Preserved properly, well labelled and deposited in a safe institution," says Brown, "these will last forever."

That is the kind of legacy to which every taxonomist aspires, and Taylor was no exception. Born in Maysville, Missouri, on 23 April 1889, he was still a teenager when he began depositing specimens at this museum. At 23, he joined the civil service and became what he called "a one-man Peace Corps" in the Philippines — then a US territory — setting up a school for members of a headhunting tribe in central Mindanao, where he collected the parachute gecko among other species. Next, he worked for the fisheries department in Manila and then completed his PhD on Philippine mammals, but his true passion was always herpetology. It came at the expense of just about everything else in his life. "I named about 500 species," he would later tell a reporter, "but I can't always remember the names of my own children." His wife, Hazel, could not bear his long absences, and they divorced in 1925.

By then, Taylor had described more species than most of his peers could achieve in a lifetime: 42 amphibians, 40 lizards and 30 snakes. He sold some of his specimens to museums in the United States, but many remained at the Bureau of Science in Manila, where he thought they would be secure forever. He joined the faculty at Kansas in 1926, and over the next two decades he wandered the globe from Mexico and Costa Rica to parts of Africa, lugging a folding army cot and subsisting on rice and evaporated milk as he collected specimens.

In his 60s, however, Taylor found himself under attack. In 1954, Robert Inger, a herpetologist at the Field Museum in Chicago, Illinois, published a withering taxonomic review of Philippine amphibians[3]. Inger, who studied only specimens in museums, axed 44 of the 87 species that Taylor had personally named or approved. "The differences between Taylor's frogs will be recognized as the differences to be expected between individuals," Inger wrote. In other words, Taylor was a hack. On his personal copy of Inger's text, Taylor scribbled the word, "Hooey."

"A LOT OF THE THINGS PEOPLE THOUGHT WERE EXTINCT, IF YOU GO RIGHT WHERE TAYLOR SAID, YOU CAN FIND THEM."

More recently, herpetologists have levelled other serious allegations against Taylor's character. In 1993, the Kansas Herpetological Society posthumously published his 1916 master's thesis on Kansas reptiles. In a foreword, one of his former students, Hobart Smith, revealed that Taylor had plagiarized large sections from the nineteenth-century palaeontologist and herpetologist Edward Drinker Cope. For those who knew Taylor as a man of principle, it was a devastating revelation, but it also explained why Taylor had never tried to publish the work himself. Then, in 2002, herpetologist Jay Savage at the University of Miami in Coral Gables, Florida, charged that Taylor had secretly copied the field notes of a rival in order to scoop him on his next collecting trip to Costa Rica[4].

Taylor had other demons. He had voiced support for eugenics programmes and reportedly refused to take on Jewish students. Brown makes no apologies for the man, but Taylor's reputation — for good or ill — is intertwined with the history of the Kansas museum. "In the end, we consider him our own," says Brown.

A LEGACY REVISITED

Brown's interest in Taylor grew when he was a graduate student at the University of Texas at Austin in the late 1990s. He devoured Taylor's monographs to plan his own collecting. He hunted through museum records to find out where Taylor's specimens were, and made visits to see them at the Field Museum and the California academy. But time and time again, he came to a dead end when he wanted information on type specimens that Taylor had deposited at the Philippine Bureau of Science.

He soon learned the tragic story of that institution: in February 1945, when US General Douglas MacArthur launched an all-out attack on Manila to expel the Japanese occupiers, the Bureau of Science was reduced to rubble, and all of its botanical and zoological specimens were destroyed, including 32 of Taylor's type specimens. "The loss is an irreplaceable one," Taylor's friend Elmer Merrill, a legendary botanist, wrote in *Science*[5]. Plant specimens were gradually replenished, but no one had systematically tried to replicate Taylor's efforts. For many years, hostile tribes kept most interlopers away from species-rich regions. In the 1990s, threats of terrorism made it difficult to access places such as the Sulu Archipelago, where Taylor collected types for a dozen species. Despite the danger, Brown resolved to retrace Taylor's steps.

In July 1998, he hired a boy to guide his team through the mountains of northern Luzon Island. It was the same place where Taylor had been ambushed by a machete-wielding native in a loincloth. While Brown tromped through streams on his quest, a rumour spread through a town below that Westerners had kidnapped the boy. A dozen locals took up torches, canes and machetes and marched to the home of the village chief on their way to find the kidnappers. When Brown returned, he diffused the situation by producing sacks of amphibians — his only captives. Taylor, when ambushed, had produced a rifle.

During the 1998 trip, Brown and his collaborators found 5 species of reptiles and amphibians not seen for many decades; 13 potentially new to science; and 30 never before reported from the region[6]. One night, Brown caught several *Platymantis* frogs making an insect-like chirp high in the trees. They turned out to be from a species that Taylor had caught and named *rivularis* in 1920. The type specimen still existed, but it was bleached of colour and in pretty bad shape, and there were not many other examples to examine. Accordingly, Inger had lumped *rivularis* in with another species, *hazelae* (named after Taylor's wife). But after hearing its mating calls and seeing its colours in life, Brown decided he would resurrect *P. rivularis* as its own species. Inger, says Brown, favoured more inclusive groupings and was draconian in his decisions. "If he had any doubt, he would sink a species."

Over the past two decades, Brown and his close collaborator Arvin Diesmos, a herpetologist at the National Museum of the Philippines in Manila, have collected more than 15,000 Philippine specimens — about one-fifth of Taylor's lifetime haul. To establish evolutionary relationships, Brown also collects DNA, which cannot be extracted from Taylor's formaldehyde-preserved specimens, and he records frog mating calls, a key tool for identifying species. By the time he has finished his own review of the Philippine *Platymantis* frogs, which has been in the works since 2003, he expects to have doubled the number of species from 30 to 60, resurrecting many of Taylor's names.

CROAK AND DAGGER

Brown's fascination with Taylor has gone beyond taxonomy. Early in his research, he became intrigued by "herpetology gossip" about Taylor's extracurricular activities. As he trotted around the globe, Taylor seemed to be conducting field work in conflict zones and, in his memoirs, he alluded to duties outside science[7]. While working for the fisheries department in Manila, he helped to investigate the murder of an Englishman, traded tips with the Swedish secret service and scouted for mercury that could be used in munitions during the First World War. On his river journeys, he occasionally noticed Japanese people, and warned the local governor that they were "spying out of the land".

It was never clear to Taylor's few confidantes whether he used wars as an excuse to get into the field, or vice versa. In his obituary, a former student suggested that Taylor's later activities during the Second World War "probably will never be known in detail"[2].

But the true nature of Taylor's work is finally coming into focus as intelligence records are declassified and research materials surface. They reveal that Taylor was indeed a spy, and that he continued to do intelligence work after the First World War, when he was sent to Siberia. His official purpose was to join the Red Cross to stop a typhus epidemic, but he was also gathering information on the Communist revolt in Russia and, later, the fate of grand duchess Anastasia, daughter of murdered tsar Nicholas II.

Taylor was called to duty again in 1944, when he was 54 and war raged in the Pacific. According to records in the US National Archives, he joined the Office of Strategic Services (OSS), a precursor to the Central Intelligence Agency (CIA), to train agents in Sri Lanka — then a British territory that provided ready access to Myanmar, Malaysia, Indonesia and other areas that the Japanese had infiltrated. Scientific work, an OSS officer explained to one of Taylor's superiors, was "excellent cover".

RICHARD GWIN/LAWRENCE JOURNAL-WORLD

Taylor taught jungle survival at Camp Y, a steamy settlement on the coast. With a penetrating stare and a lantern jaw, he seemed more imposing than his 1.8 metres. In his spare time, he occasionally dodged gunfire to nab specimens, which he studied for two monographs published after the war. "Have just described five new forms of blind snakes from the island," he wrote to S. Dillon Ripley, a young ornithologist who served with him and would later lead the Smithsonian Institution in Washington DC. In a later letter, he offered "some 500 species" of mollusc shells to the Smithsonian.

After the war, Taylor helped the British in Malaysia to investigate Japanese war crimes against civilians. His work documenting rape, torture and murder may have contributed to his antipathy towards the Japanese people. Never an easy-going person, his experiences at war seem to have wounded him. He failed in a bid to become head of the Kansas museum, and grew increasingly paranoid in daily life. He studied Russian and made inquiries about working for the CIA. Smith, who died in March this year, told *Nature* that Taylor sprinkled flour on the floor of his office to detect trespassers during his absences. "I was wary of him," said Smith. William Duellman, a herpetologist at the University of Kansas who first met Taylor in 1951, thinks that Taylor's symptoms could today meet the standards of post-traumatic stress disorder. Nevertheless, Taylor kept working. In his later years, he studied a group of poorly known, legless amphibians called caecilians. He published a sprawling, 800-page taxonomic review[8] of them in 1968.

Rafe Brown holds a specimen of an adult snake, collected by Edward Taylor in the 1920s.

ON THE BRINK

Taylor's herpetological legacy in the Philippines has taken on new importance now that the country has lost more than 95% of its native forest. Species collectors such as Brown know that their work has conservation implications, but there are often differences between scientific studies and conservation classifications. In the late 1990s, for example, the International Union for Conservation of Nature (IUCN) labelled the Polillo Island frog — *Platymantis polillensis*, first described by Taylor — as critically endangered. All but 4 square kilometres of Polillo's forests had been razed for coconut plantations.

But in 2004, Brown was listening to his recordings when he noticed that the Polillo frog had a mating call similar to that of a frog that he had collected on Luzon. Brown applied for permission to get genetic samples from Taylor's original collecting site, and confirmed his hunch: the frog is widespread. Last year, he reported[9] that seven frog species once considered vulnerable or endangered by the IUCN are actually widespread on Luzon.

The challenge for taxonomists is that although many agree that global biodiversity is in crisis, threat levels are hard to gauge accurately because advocates for every taxon and ecosystem are clamouring for attention and real data are scarce. "Global threat assessments for large taxonomic groups is a very inexact science," says Walter Jetz, an ecologist at Yale University in New Haven, Connecticut. "We need more boots on the ground."

Brown is sceptical about conservation assessments in general, but one threat to Philippine amphibians does concern him: the chytrid fungus *Batrachochytrium dendrobatidis*, which has been linked to the decline or extinction of hundreds of amphibian species around the world (see *Nature* **465**, 680–681; 2010). In 2009, Brown identified the fungus on five species in the Philippines, and it has since been found on more. The chytrid threat, he says, combined with habitat destruction and climate change, could push Philippine amphibians over the edge.

Time is running out to document the biodiversity of the Philippines, but also to determine Taylor's place in history. Brown has found that Taylor's species descriptions, although brief, often zeroed in on the precise trait that set one group apart from its relatives. "He had a sharp eye," says Brown. More than a dozen species whose names were erased by Inger and others have proved to be valid after all.

Inger, who is 93, is impressed by the emerging evidence and the way that Brown has approached the subject. "I think he's probably right," he says, but adds, "I'm still a little uneasy about over-fragmentation."

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For a podcast and slideshow about Taylor and Brown, see:
go.nature.com/hnforn

Back at the University of Kansas, Brown takes a seat inside an archival library and dips once more into some of Taylor's work, including the battered leather books that the man used for his field notes and specimen catalogues. Paging through one of those catalogues for the first time, Brown is stunned to find that Taylor had crossed out the name attached to an Asian spadefoot toad that he caught on Mindoro Island — a strange, gangly creature that crawls rather than hops. Next to it, Taylor had written, "new sp!!". As recently as 2009, Brown had designated it as a new species, *Leptobrachium mangyanorum*, because it was so different from previously described relatives[10].

"Ed was way ahead of us," says Brown. "Why he never named it, we'll never know. But it's pretty satisfying to come along 90 to 100 years later and arrive at the same conclusion." ■

Brendan Borrell *is a biologist-turned-journalist based in New York. He contributed to a 2007 review paper on gliding animals, which also included work by Brown.*

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QUANTUM QUEST

PHYSICISTS HAVE SPENT A CENTURY PUZZLING OVER THE PARADOXES OF QUANTUM THEORY. NOW A FEW OF THEM ARE TRYING TO REINVENT IT.

BY PHILIP BALL

If the truth be told, few physicists have ever really felt comfortable with quantum theory. Having lived with it now for more than a century, they have managed to forge a good working relationship; physicists now routinely use the mathematics of quantum behaviour to make stunningly accurate calculations about molecular structure, high-energy particle collisions, semiconductor behaviour, spectral emissions and much more.

But the interactions tend to be strictly formal. As soon as researchers try to get behind the mask and ask what the mathematics mean, they run straight into a seemingly impenetrable wall of paradoxes. Can something really be a particle and a wave at the same time? Is Schrödinger's cat really both alive and dead? Is it true that even the gentlest conceivable measurement can somehow have an effect on particles halfway across the Universe?

Many physicists respond to this inner weirdness by retreating into the 'Copenhagen interpretation' articulated by Niels Bohr, Werner Heisenberg and their colleagues as they were putting quantum theory into its modern form in the 1920s. The interpretation says that the weirdness reflects fundamental limits on what can be known about the world, and just has to be accepted as the way things are — or, as famously phrased by physicist David Mermin of Cornell University in Ithaca, New York, "shut up and calculate!"[1]

But there have always been some who are not content to shut up — who are determined to get behind the mask and fathom quantum theory's meaning. "What is it about this world that forces us to navigate it with the help of such an abstract entity?" wonders physicist Maximilian Schlosshauer of the University of Portland in Oregon, referring to the uncertainty principle; the wave function that describes the probability of finding a system in various states; and all the other mathematical paraphernalia found in textbooks on quantum theory.

Over the past decade or so, a small community of these questioners have begun to argue that the only way forward is to demolish the abstract entity and start again.

ILLUSTRATION BY ANDY POTTS

They are a diverse bunch, each with a different idea of how such a 'quantum reconstruction' should proceed. But they share a conviction that physicists have spent the past century looking at quantum theory from the wrong angle, making its shadow odd, spiky and hard to decode. If they could only find the right perspective, they believe, all would become clear, and long-standing mysteries such as the quantum nature of gravity might resolve themselves in some natural, obvious way — perhaps as an aspect of some generalized theory of probability.

"The very best quantum-foundational effort," says Christopher Fuchs of the Perimeter Institute for Theoretical Physics in Waterloo, Canada, "will be the one that can write a story — literally a story, all in plain words — so compelling and so masterful in its imagery that the mathematics of quantum mechanics in all its exact technical detail will fall out as a matter of course".

A VERY REASONABLE PROPOSAL

One of the earliest attempts to tell such a story came in 2001, when Lucien Hardy, then at the University of Oxford, UK, proposed that quantum theory might be derived from a small set of "very reasonable" axioms about how probabilities can be measured in any system[2], such as a coin tossed into the air.

Hardy began by noting that a classical system can be specified completely by measuring a certain number of 'pure' states, which he denoted N. For a coin toss, in which the result is always either heads or tails, N equals two. For the roll of a dice, whereby the cube must end up with one of six faces uppermost, N equals six.

Probability works differently in the quantum world, however. Measuring the spin of an electron, for example, can distinguish two pure states, which can be crudely pictured as a rotation clockwise or anticlockwise around, say, a vertical axis. But, unlike in the classical world, the electron's spin is a mixture of the two quantum states before a measurement is made, and that mixture varies along a continuum. Hardy accounted for that through a 'continuity axiom', which demands that pure states transform from one to another in a smooth way. This axiom turns out to imply that at least N^2 measurements are required to completely specify a system — a relationship that corresponds to the standard quantum picture.

But, in principle, said Hardy, the continuity axiom also allows for higher-order theories in which a complete definition of the system requires N^3, N^4 or more measurements[3], resulting in subtle deviations from standard quantum behaviour that might be observable in the lab. He did not attempt to analyse such possibilities in any detail, however; his larger goal was to show how quantum physics might be reframed as a general theory of probability. Conceivably, he says, such a theory could have been derived by nineteenth-century mathematicians without any knowledge of the empirical motivations that led Max Planck and Albert Einstein to initiate quantum mechanics at the start of the twentieth century.

Fuchs, for one, found Hardy's paper electrifying. "It hit me over the head like a hammer and has shaped my thinking ever since," he says, convincing him to pursue the probability approach wholeheartedly.

Fuchs was especially eager to reinterpret the troubling concept of entanglement: a situation in which the quantum states of two or more particles are interdependent, meaning that a measurement of one of them will instantaneously allow the measurer to determine the state of the other. For example, two photons emitted from an atomic nucleus in opposite directions might be entangled so that one is polarized horizontally and the other is polarized vertically. Before any measurement is made, the polarizations of the photons are correlated but not fixed. Once a measurement on one photon is made, however, the other also becomes instantaneously determined — even if it is already light years away.

As Einstein and his co-workers pointed out in 1935, such an instantaneous action over arbitrarily large distances seems to violate the theory of relativity, which holds that nothing can travel faster than light. They argued that this paradox was proof that quantum theory was incomplete.

But the other pioneers stood fast. According to Erwin Schrödinger, who coined the term 'entanglement', this feature is the essential trait of quantum mechanics, "the one that enforces its entire departure from classical lines of thought". Subsequent analysis has resolved the paradox, by showing that measurements of an entangled system cannot actually be used to transmit information faster than light. And experiments on photons in the 1980s showed that entanglements really do work this way.

Still, this does seem an odd way for the Universe to behave. And this is what prompted Fuchs to call for a fresh approach to quantum foundations[4]. He rejected the idea, held by many in the field, that wave functions, entanglement and all the rest represent something real out in the world (see *Nature* **485,** 157–158; 2012). Instead, extending a line of argument that dates back to the Copenhagen interpretation, he insisted that these mathematical constructs are just a way to quantify "observers' *personal* information, expectations, degrees of belief"[5].

He is encouraged in this view by the work of his Perimeter Institute colleague Robert Spekkens, who carried out a thought experiment asking what physics would look like if nature somehow limited what any observer could know about a system by imposing a "knowledge balance principle": no observer's information about the system, as measured in bits, can ever exceed the amount of information he or she lacks. Spekkens' calculations show that this principle, arbitrary as it seems, is sufficient to reproduce many of the characteristics of quantum theory, including entanglement[6]. Other kinds of restriction on what can be known about a suite of states have also been shown to produce quantum-like behaviours[7,8].

KNOWLEDGE GAP

The lesson, says Fuchs, isn't that Spekkens's model is realistic — it was never meant to be — but that entanglement and all the other strange phenomena of quantum theory are not a completely new form of physics. They could just as easily arise from a theory of knowledge and its limits.

To get a better sense of how, Fuchs has rewritten standard quantum theory into a form that closely resembles a branch of classical probability theory known as Bayesian inference, which has its roots in the eighteenth century. In the Bayesian view, probabilities aren't intrinsic quantities 'attached' to objects. Rather, they quantify an observer's personal degree of belief of what might happen to the object. Fuchs' quantum Bayesian view, or QBism (pronounced 'cubism')[9,10], is a framework that allows known quantum phenomena to be recovered from new axioms that do not require mathematical constructs such as wavefunctions. QBism is already motivating experimental proposals, he says. Such experiments might reveal, for example, new, deep structures within quantum mechanics that would allow quantum probability laws to be re-expressed as minor variations of standard probability theory[11].

"That new view, if it proves valid, could change our understanding of how to build quantum computers and other quantum-information kits," he says, noting that all such applications are critically dependent on the behaviour of quantum probability.

Knowledge — which is typically measured in terms of how many bits of information an observer has about a system — is the focus of many other approaches to reconstruction, too. As physicists Časlav Brukner and Anton Zeilinger of the University of Vienna put it, "quantum physics is an elementary theory of information"[12]. Meanwhile, physicist Marcin Pawłowski at the University of Gdańsk in Poland and his colleagues are exploring a principle they call 'information causality'[13]. This postulate says that if one experimenter (call her Alice) sends m bits of information about her data to another observer (Bob), then Bob can gain no more than m classical bits of information about that data — no matter how much he may know about Alice's experiment.

Pawłowski and his colleagues have found that this postulate is respected by classical physics and by standard quantum mechanics, but not by alternative theories that allow for stronger forms of entanglement-like correlations between information-carrying particles. For that reason, the group writes in their paper, "information causality might be one of the foundational properties of nature" — in other words, an axiom of some future, reconstructed quantum theory.

"A LOT OF THE FEATURES WE THINK OF AS UNIQUELY QUANTUM ARE GENERIC TO MANY PROBABILISTIC THEORIES."

What is striking about several of these attempts at quantum reconstruction is that they suggest that the set of laws governing our Universe is just one of many mathematical possibilities. "It turns out that many principles lead to a whole class of probabilistic theories, and not specifically quantum theory," says Schlosshauer. This is in itself a valuable insight. "A lot of the features we think of as uniquely quantum," he says, "are actually generic to many probabilistic theories. This allows us to focus on the question of what makes quantum theory unique."

POISED FOR SUCCESS?

Hardy says that the pace of quantum-reconstruction efforts has really picked up during the past few years as investigators begin to sense they are getting some good handles on the issue. "We're now poised for some really significant breakthroughs," he says.

But how can anyone judge the success of these efforts? Hardy notes that some investigators are looking for experimental signs of the higher-order quantum correlations allowed in his theory. "However, I would say that the real criterion for success is more theoretical," he says. "Do we have a better understanding of quantum theory, and do the axioms give us new ideas as to how to go beyond current-day physics?" He is hopeful that some of these principles might eventually assist in the development of a theory of quantum gravity.

There is plenty of room for scepticism. "Reconstructing quantum theory from a set of basic principles seems like an idea with the odds greatly against it," says Daniel Greenberger, a physicist who works on quantum foundations at the City College of New York[5]. Yet Schlosshauer argues that "even if no single reconstruction program can actually find a universally accepted set of principles that works, it's not a wasted effort, because we will have learned so much along the way".

He is cautiously optimistic. "Once we have a set of simple and physically intuitive principles, and a convincing story to go with them, quantum mechanics will look a lot less mysterious", he says. "I think a lot of the outstanding questions will then go away. I'm probably not the only one who would love to be around to witness the discovery of these principles." ■

Philip Ball *is a freelance writer based in London.*

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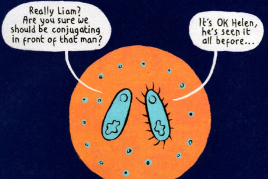
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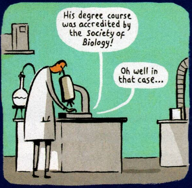
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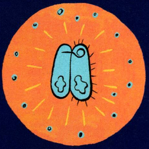

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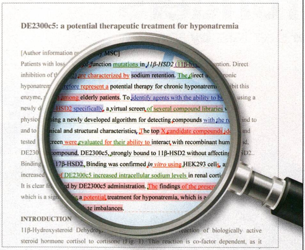

DE2300c5: a potential therapeutic treatment for hyponatremia

[Author information r y MSC]
Patients with loss of function mutations in 11β-HSD2 (11β-hy ention. Direct
inhibition of t are characterized by sodium retention. The direct i ronic
hyponatrem refore represent a potential therapy for chronic hyponatrem ibit this
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DE230 compound, DE2300c5, strongly bound to 11β-HSD2 without affecting SD2.
Binding 17β-HSD2. Binding was confirmed in vitro using HEK293 cells, a
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It is clear fr d by DE2300c5 administration. The findings of the presen
which is a sig a potential treatment for hyponatremia, which is a
te imbalances.

INTRODUCTION
11β-Hydroxysteroid Dehydrog eaction of biologically active
steroid hormone cortisol to cortisone (Fig. 1). This reaction is co-factor dependent, as it

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PHYSICS Film and memoir drop intriguing hints about Stephen Hawking **p.162**

EVOLUTION First volume of Richard Dawkins' memoir reviewed **p.163**

NEIL WEBB

A single market for European research

European collaboration is not far behind that in the United States, but there is still work to be done on cross-border funding and financial inequalities, says **Paul Boyle**.

Building the European Research Area (ERA) has been the priority of Máire Geoghegan-Quinn since her appointment as the European Commissioner for Research, Innovation and Science in 2009. Her mandate stipulates that the ERA should "ensure the free circulation of researchers, knowledge, ideas and technology" across the European Union (EU; see go.nature.com/qdlyri) — like an academic equivalent of the European single market for goods and services. In March 2012, the European Council called for it to be completed by 2014 (see go.nature.com/meukwn). One focus, of particular relevance to the national funding agencies, has been on increasing researcher collaboration and mobility within Europe.

As president of Science Europe (representing 53 research-funding and -performing organizations from 27 countries) and chief executive of the UK Economic and Social Research Council (ESRC), I believe that the proposed timetable for implementing the ERA is worryingly short, particularly if changes to funding agencies and other institutional practices are necessary. I am also concerned that the changes may produce some undesirable, unintended consequences.

To agree on how much collaboration is optimal, we need to know how researchers collaborate and move within Europe. At Science Europe's request, such an analysis, produced by scientific publisher Elsevier's SciVal Analytics Team, will be released this week[1]. The report shows that internal European collaboration is not far behind that in the United States, but that connections with countries outside Europe need nurturing too. Researcher mobility between institutions in different European countries is also relatively low compared with movement between US states. To further the ERA vision, we

▶

should promote best practices for cross-border funding and minimize inequalities in salaries, pensions and benefits.

In Europe, most scientific research is supported by national agencies, rather than through European or international programmes. Different agencies take different approaches to collaboration. Some allocate resources across borders: the ESRC, for example, allows up to 30% of any grant to be spent on international collaborations. By contrast, many agencies restrict their spending to their home nations, and legislation to alter this requirement would be impossible to attain by 2014, even if they wished to do so.

Some organizations pay proportionately for researchers from their country in transnational projects. One approach is that collaborators submit a proposal to a single 'lead agency', which reviews the bid and takes the funding decision. Agencies from other countries recognize the decision and pay their share. Although this is efficient, negating the need for peer review in multiple nations, problems can arise. Committing high proportions of an agency's budget on the basis of decisions taken in another nation could lead to local funding panels feeling deprived of power. Lead-agency agreements work best when those involved have similar peer-review standards, success rates and views on research priorities. Agreements cannot be imposed on unwilling partners.

STATE OF THE UNION

Comparisons are often made with the greater level of partnership across US states (see go.nature.com/w87clf). But, unlike Europe, the US states are part of a federal system, with one language, consistent labour-market conditions and a single national funding system.

To provide a benchmark, Science Europe asked Elsevier to draw on publication data from their bibliographic database Scopus on the level of collaboration and mobility in Europe and the United States. The report[1] will be launched in Brussels on 16 September.

The analysis found that, in 2011, 13% of papers with a European primary author included co-authors from more than one country in Europe, compared with 16% of US primary-author papers that involved inter-state collaborations (see 'Affiliation trends'). This difference is small. But it is intercontinental collaborations — those that involve authors outside Europe or the United States, respectively — that tend to produce the most highly cited papers. So it is concerning that European scientists have fewer such partnerships (23% of joint papers) than their US counterparts (30%).

> ***"Few academics seem to move between countries in Europe."***

Relatively few academics seem to move between countries in Europe. The report finds that between 1996 and 2011, only 7% of researchers' affiliations switched between European countries, according to their addresses. In the United States, 22% of researchers published from institutions in more than one state in the same period. Barriers to movement across European borders might include language, benefits systems and cultural differences.

Although many funding agencies cannot legally allocate their resources to another nation, some have agreed a 'money follows researcher' policy, allowing researchers to transfer grants if they relocate within Europe. But even this simple scheme has challenges.

Who owns the intellectual property from the grant-funded work when a researcher moves? Why would an academic move to a country where salaries or pensions are lower? How long would a national agency support such a policy if several researchers and their grants emigrated but few immigrated? And would the strongest researchers concentrate in the best-performing countries and institutions, perhaps increasing European excellence overall, but punishing nations that are still building their science portfolios?

Progress on various fronts is required[2]. First, regular monitoring of researcher collaborations and movements in Europe is needed. Funding agencies, universities and the EU should work together to collect different parts of this information.

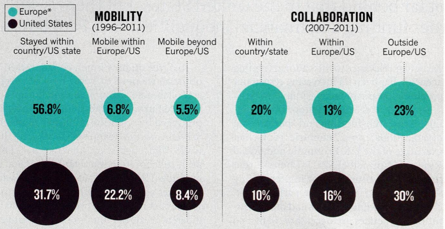
Second, best practices, drawn from the various schemes, should be adopted across Europe. These should be administratively simple and avoid double peer review, where possible. Clarity is required on terminology — even words such as 'grant' or 'evaluation' can mean different things in different organizations — and on how funding mechanisms are implemented and communicated to researchers. Science Europe's working group on cross-border collaboration is now preparing such guidance.

Third, we need a forum that brings together research-funding and -performing organizations, the European Commission and ministerial representatives from member states. Science Europe has committed to hosting such a high-level ERA workshop annually.

Fourth, European funding agencies should not overemphasize European collaboration at the expense of global partnerships. Best-with-best partnerships should be encouraged, wherever they might be.

Fifth, research-funding and -performing organizations, through Science Europe, and universities must work with the European Commission to identify and solve barriers to mobility in the labour market, welfare and administrative systems. These include issues such as pensions portability, coordination of social-security systems and transparent recognition of educational qualifications.

The ERA should be an evolving, flexible and creative space in which researchers, ideas and knowledge circulate freely to respond to society's challenges. At its heart will be trust. The establishment of Science Europe is itself testament to the willingness of European research agencies to engage in shaping a better research landscape. ■



AFFILIATION TRENDS

Researcher mobility and collaboration, revealed though publication data, are lower across Europe than across the United States, in part owing to language and cultural barriers.

*Percentages exclude transitory mobility and single-author/-institute papers. Europe refers to the 41 countries eligible for Seventh Framework Programme funding: 27 EU member states and 14 associated countries.

SOURCE: REF. 1

Paul Boyle *is chief executive of the UK Economic and Social Research Council in Swindon, UK, and president of Science Europe in Brussels.*
e-mail: esrc.ceo@esrc.ac.uk

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PAUL HACKETT/IN PICTURES/CORBIS

The purported decline in bee numbers raises questions about evidence quality.

A standard for policy-relevant science

Ian Boyd calls for an auditing process to help policy-makers to navigate research bias.

The increasing concern about unreliability in scientific literature[1,2] is a problem for people like me — I am the science adviser to DEFRA, the UK government department for environment, food and rural affairs. To counsel politicians, I must recognize systematic bias in research. Bias is cryptic enough in individual studies, let alone in whole bodies of literature that contain important inaccuracies[2,3].

It worries me that because of bias, some parts of the published scientific literature, such as studies on the safety of genetically modified (GM) organisms and pesticides, or trends in biodiversity measurements, might have only limited use in policy-making.

To mitigate this problem, policy-makers should consider holding published scientific evidence to an audited standard that can be replicated and is robust to variations in assessor competence. A weighting factor, or 'kite mark', applied to journals or individual articles, could help policy-makers to assess the robustness of studies for use in particular applications. Similar methods established by non-profit standards associations are used in research to certify laboratory practice and in engineering to certify building standards.

The quality of research results fluctuates because of varying tractability in the problems being probed[1,4]. For example, it is easy to judge the efficacy of an experiment to engineer a tomato to produce the pigment anthocyanin[5], because if it succeeds, that tomato is the colour of a ripe plum. It is much harder to judge the reliability of a study investigating whether a GM crop is toxic to animals[6]. The latter situation is much more susceptible to inaccuracy and interpretation.

These problems are amplified in complex issues such as the environmental effects of GM organisms or chemical pollutants, including pesticides and endocrine disrupters. In these cases, experimentation is needed at scales large enough to provide statistical power in the presence of high background noise. The problem is amplified further when statistical inference is used.

SCOPING THE PROBLEM

Systematic bias across whole fields of science is even more cryptic and therefore more problematic. It could stem from the combined effects of how science is commissioned, conducted, reported and used, and also from how scientists themselves are incentivized to conduct certain research[7]. Such bias results from actively searching for a particular outcome, rather than performing balanced hypothesis testing. For example, in 2006, researchers in the United Kingdom and in the Netherlands found that the number of insect pollinators might have declined[8]. A consequent call for proposals (see go.nature.com/audhny) contained the underlying assumption that there was a decline, rather than conveying a need to establish whether current information about declines was robust.

Another problem is the tendency to treat different studies as statistically independent, even when they have emerged from connected commissioning processes and could therefore amount to multiple testing of the same hypothesis, meaning that every extra study must overcome an increasingly rigorous statistical hurdle to demonstrate efficacy. In combination, these kinds of bias can make individual or groups of studies that report certain effects seem more important than they really are. I suspect that these effects could be a factor in the continuing controversies surrounding genetic modification and the failure of the EU regulatory system to process applications to license new GM products.

A common reaction to such controversy is to commission subject reviews or meta-analyses[1] that assess the weight of evidence for certain effects across many individual studies. Ideally, reviewers would use processes similar to those deployed in the Cochrane Reviews that inform decision-making in health care[9].

But reviews also contain pitfalls. First, they risk amplifying rather than eliminating systematic bias — which could be more common in some subjects than others. Second, they can be affected by the increasing tendency not to publish 'negative' results[4]. Meta-analyses can compound the prevalence of false positives in the literature, and can be blind to unreported true negatives. We need rules for how to deal with these issues when compiling literature reviews for policy-relevant research.

SEAL OF APPROVAL

Strict procedures govern experimental design and the evidence standards for trials that are used to determine the efficacy and safety of GM organisms, pesticides or drug therapies. But once products are licensed for use, they are often subject to less formal investigations. The same relaxation of rules applies to testing the efficacy of policy interventions. Ad hoc studies, with all the problems outlined above, can then carry disproportionate political weight when their results question the operational integrity of a licensed product, or the effectiveness of a policy[10]. Quality-control criteria are needed for these studies that are outside a regulatory framework.

We need an international audited standard that grades studies, or perhaps journals. It would evaluate how research was commissioned, designed, conducted and reported. This audit procedure would assess many of the fundamental components of scientific studies, such as appropriate statistical power; precision and accuracy of measurements; and validation data for assays and models. It would also consider conflicts of interest, actual or implied, and more challenging issues about the extent to which the conclusions follow from the data. Any research paper or journal that does not present all the information needed for audit would automatically attract a low grade.

> ***"What I propose augments rather than replaces peer review."***

Such a system would provide policy officials and others with a reliable way of assessing evidence quality, and it would drive up standards in scientific research to reverse the worrying trends that suggest underlying bias[1–4,7].

Critics will counter that my proposed certification standard would be subjective and would shift the job of assessing quality away from expert peer reviewers. But in its current form, peer review fails to set a consistent standard. What I propose augments rather than replaces peer review, and assessment could be carried out on behalf of authors, journals or users of information through the use of third-party certified auditors.

I do not underestimate the challenge of establishing such a system, but it would bring standards to scientific publishing that are common practice in other disciplines. Ultimately, this will increase the rigour and transparency around the scientific literature that is used in policy decisions. ■

Ian Boyd *is chief scientific adviser at the UK Department of Environment, Food and Rural Affairs in London. He is also professor in biology at the University of St Andrews, UK.*
e-mail: ian.boyd@defra.gsi.gov.uk

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Bring on the evidence

It is time to probe whether the trend for patient and public involvement in medical research is beneficial, say **Sophie Petit-Zeman** and **Louise Locock**.

Involving patients and the public as partners in medical research — from deciding what to study to influencing how results are used — is an emerging force. For some, the approach is based on common sense and justice[1]. Others, such as the chief medical officer for England, Sally Davies, feel that the advice of patients and the public "invariably makes studies more effective, more credible and often more cost efficient"[2].

The Seventh Framework Programme (FP7), the European Union's current research-funding instrument, stresses[3] the importance of patient and public involvement, known as PPI. And the Patient-Centered Outcomes Research Institute in Washington DC has allocated US$68 million to a research network predicated on the principle that "the interests of patients will be central to decision-making" (see go.nature.com/mdhy6i).

PPI is a prerequisite for much UK government research funding and it is spreading among funders, health-care organizations and charities[4]. The James Lind Alliance (JLA), with which one of us (S.P-Z.) has worked since its inception in 2004, enables patients, carers and clinicians to agree on what research matters most. It explicitly excludes the pharmaceutical industry and pure researchers. After a decade of arms-length government support, the JLA is now part of the National Institute for Health Research (NIHR) based in Southampton, UK, and JLA partnerships are complete or underway for 25 medical conditions (see go.nature.com/twhvxz). For example, the NIHR Oxford Biomedical Research Centre is running partnerships in spinal-cord injury and joint-replacement surgery, and it is the first major research institution to be appointing staff to use the JLA method 'in house', closing the loop between what matters to patients and what is researched in their name.

This international growth of PPI is rightly paralleled by unease at the paucity of evidence for its impact. And the evidence there is, including the findings that PPI improves recruitment to studies and changes what is researched[2,5], is weak. As Simon Denegri, the United Kingdom's first national director for public participation and engagement in research, put it: "The evidence-base for PPI's impact is meagre, patchy and largely observational."

SELF-EXAMINATION

Those of us working in PPI must robustly examine our own practices with a common set of tools. Otherwise, we will struggle to answer PPI sceptics, such as one researcher who asked: "Why should patients have useful opinions about what directions research should take?"[6].

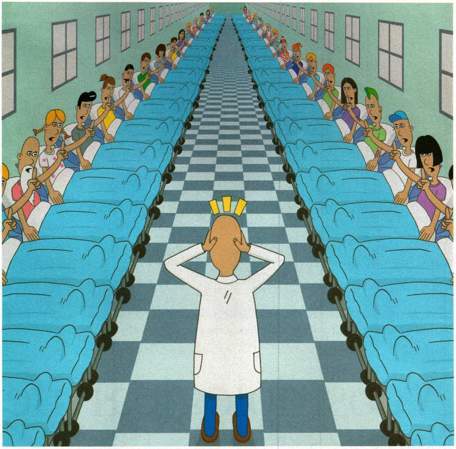

ILLUSTRATION BY CHRIS RYAN/NATURE

A first crucial step is to ensure consistent, accurate reporting of what PPI has been done and how. We can assess whether an activity is useful only if it is clear what it was.

This challenge is being addressed through GRIPP[7] (Guidance for Reporting Involvement of Patients and Public), a checklist published in 2011 for studies that include PPI to help authors and readers to critically appraise the work. GRIPP is being used to generate consensus through the EQUATOR Network, an international initiative that promotes the development and spread of guidelines for health-research reporting[4].

A key element of reporting PPI is to make clear who was involved, in part to allow us to gauge when it matters to distinguish between public and patient input. One demonstrable effect of PPI is that it helps to create user-friendly information, questionnaires and interview schedules for patients[5]. But this sort of reality check about jargon differs from gathering and heeding patient experiences.

We must also probe whether PPI is valuable for all research types. Will it ever, for example, have a place in basic science? Anecdotal evidence suggests that it might, in part because patients push for research into causes. The UK Alzheimer's Society, the only funder that works with people with dementia and their carers to select research projects, backs work from the lab bench to the clinic. And priorities in sight-loss research, identified through the JLA approach, revealed patient interest in causation, as well as treatments using stem cells and gene therapy.

One of the knottiest problems in PPI is how to best weigh up anecdotes and evidence. How are the patients involved chosen? Do they bring more than their own views? Are diverse voices heard, or just those that are loudest?

Ignore such questions, and PPI might unwittingly perpetuate power imbalances. Patients can achieve involvement through existing networks, but not all will be part of these, or they might be chosen by a researcher who is keen to work with a kindred spirit. The most well-meaning approaches can simply extend input from educated, middle-class professionals to input from educated, middle-class patients.

Yet we must also avoid double standards. Just as people will always want the best researchers or clinicians, we must not exclude the most informed or articulate patients[8].

There is no easy fix, but the ability of involved patients to represent wider views can be optimized through routes such as the website www.healthtalkonline.org, led by the University of Oxford's Health Experiences Research Group (HERG), where one of us (L.L.) is deputy research director.

Healthtalkonline and its sister site, www.youthhealthtalk.org, contain video, audio and written records of nearly 3,000 people's experiences of more than 75 health-related issues. The websites allow patients and professionals to broaden their knowledge of what it is like to be ill or to make difficult health-care decisions.

Using qualitative research, interviews with subjects continue until no major new themes emerge, indicating that a comprehensive set of views has been gathered. As Sue Ziebland, HERG's research director, explains: "Supporting patients involved in research to draw from a pool of views helps defend them from accusations that they bring only their own agenda."

BUILDING A CASE

Gathering the evidence base for PPI will take time. The methodological issues described here must be addressed, and the crucial question — whether research using PPI makes life better for patients — is complex. A project funded by the UK Medical Research Council last week launched its Public Involvement Impact Assessment Framework, a resource to support research teams to develop impact-assessment tools appropriate for their work.

As PPI matures, we must find ways to ensure that those who do it, be they professionals, patients or public participants, are offered support and training — perhaps most crucially to help them to understand each others' worlds. We must then report, dissect and assess involvement, devising impact measures with patients as partners, in ways that optimize the potential of patient-centred science. ■

Sophie Petit-Zeman *is director of patient involvement in research at the NIHR Oxford Biomedical Research Centre, Oxford University Hospitals NHS Trust, UK.*
Louise Locock *is deputy research director, Health Experiences Research Group, Department of Primary Care Health Sciences, University of Oxford, UK.*
e-mail: sophie.petit-zeman@ouh.nhs.uk.

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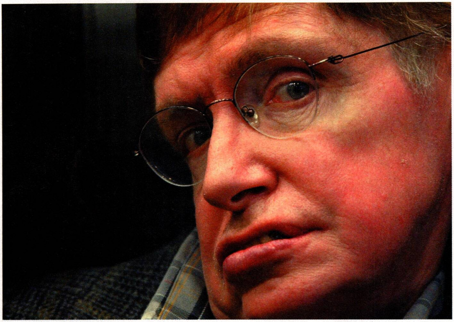
The views expressed are those of the authors and not necessarily those of the National Health Service, the National Institute for Health Research or the UK Department of Health. The authors declare competing financial interests: see go.nature.com/jedv2a for details.



Stephen Hawking, the subject of both a new memoir and a documentary film.

MIGUEL RIOPA/AFP/GETTY

PHYSICS

A cosmological life

Robert P. Crease weighs up two takes on Stephen Hawking — including the theoretical physicist's own.

True to its name, Stephen Hawking's autobiography *My Brief History* is a model of brevity, at just 20,000 words. A new documentary about the renowned theoretical physicist, *Hawking*, takes longer to watch than the book does to read. These separate projects add little to our understanding of Hawking, but do feed our insatiable curiosity about him.

Both vehicles do a creditable job of reviewing the outlines of Hawking's story, which is a compelling one. In 1963, while a graduate student at the University of Cambridge, UK, Hawking learned that he had motor neuron disease (known in the United States as Lou Gehrig's disease). In 1970, he began work on the theory of black holes, predicting in 1974 that they emit radiation and could therefore potentially evaporate: 'Hawking radiation' is probably his most significant scientific contribution. Gradually losing mobility and speech, he survived thanks to an indomitable spirit, devoted assistants and increasingly sophisticated technology. (He wrote the memoir using a sensor attached to his glasses that responds to cheek-muscle twitches.) Hawking's *A Brief History of Time* (Bantam, 1988) is surely the most popular science book ever. And he has become, to the public, the greatest living scientist — as well as a media magnet who enjoys making outrageous claims ("Philosophy is dead").

My Brief History
STEPHEN HAWKING
Bantam: 2013.

Hawking
DIRECTED BY STEPHEN FINNIGAN
Cambridge Film Festival, UK. 19 September 2013.

Meanwhile, Hawking has a solid, if not superhuman, reputation among scientists. In 1999, a *Physics World* survey asked eminent physicists to name the five physicists they thought had made the most significant contributions. Of the 61 named — 11 of them living — Hawking received only one vote.

My Brief History does not do what we expect of a memoir. It does not take the reader behind any scenes. Hawking narrates his life non-introspectively, celebrating its triumphs and burnishing its sensitive moments. It is a concise, gleaming portrait, not unlike those issued by the public relations department of an institution. "Not knowing what was going to happen to me or how rapidly the disease would progress, I was at a loose end," he writes of his reaction to being diagnosed. Nor is he reflective about his relationships with his ex-wives Jane Wilde and Elaine Mason. "My marriage to Elaine was passionate and tempestuous," he writes. "We had our ups and downs."

The book provides no revelations, deep insight, messy details or score-settling, and does not explore his celebrity status. Hints of emotion are rare. At one point he recalls thinking himself a tragic figure, and begins to listen to Wagner. At another he remembers seeing a boy in the hospital bed next to him die of leukaemia. "Whenever I feel inclined to be sorry for myself, I remember that boy." You sense something like a soul behind Hawking's dispassionate account, but, like a black hole, its existence has to be deduced from external indications.

Hawking, directed by Stephen Finnigan, is a good celebrity biopic. In it, Hawking narrates many of the same events as in *My Brief History*, often in the same words. But the film is diverting in a way that the book is not: we get to see clips of Hawking's appearances as himself or in cartoon form on US television shows such as *Star Trek* and *The Simpsons*. We hear Wagner and Pink Floyd on the soundtrack, watch re-enactments of episodes from Hawking's early life, and see a snapshot of Hawking with President Obama and First Lady Michelle.

Hawking's synthesized voice can lend an aura of gravitas to words that would come off as platitudes on the page. "When you are faced with the possibility of an early death, it makes you realize that life is worth living", he says. Occasionally, even the synthesizer cannot rescue his pronouncements from sounding naive: "Sometimes I wonder if I am as famous for my wheelchair and disabilities as I am for my discoveries." The film concludes with spectacular footage from the 2012 Paralympics in London, at which Hawking opened the ceremonies.

These two offerings add to a growing list that do offer new knowledge about the cosmologist. Jane Hawking's *Travelling to Infinity* (Alma Books, revised, 2008) divulges aspects of their relationship. Kitty Ferguson's *Stephen Hawking: An Unfettered Mind* (Palgrave Macmillan, 2012) explores his life and illness. Hélène Mialet's *Hawking Incorporated* (University of Chicago Press, 2012) analyses the networks of people, media and technologies without which there would be no Stephen Hawking as we know him.

Mialet demolishes the popular myth of Hawking as a solitary genius. But it is an irresistible one. "Thank you for coming on a journey through my world," Hawking says at the conclusion of the documentary. In truth, *Hawking* is a carefully stage-managed tour of only part of that world, yet it is a skilful, entertaining and moving trip nonetheless. ■

Robert P. Crease *is professor of philosophy at Stony Brook University, New York, and author of* World in the Balance.
e-mail: robert.crease@stonybrook.edu

BIOLOGY

Evolution of a mind

Eugenie C. Scott revels in the first volume of Richard Dawkins's frank new memoir.

Richard Dawkins is one of the world's best-known scientists — largely because of his tireless promotion of evolution and, more recently, atheism, through a succession of best-sellers, videos and public appearances. In *An Appetite for Wonder*, the first part of a two-volume memoir, Dawkins presents his life up to the publication of *The Selfish Gene* (Oxford University Press, 1976), the popular-science blockbuster that kick-started his career as a spokesperson for evolutionary biology.

As befits someone whose life's work has focused on the cumulative changes through time that we call evolution, Dawkins wants to share the forces and factors in his life that have shaped the person he has become. A bookish child, he neglected many opportunities provided by his nature-loving parents to explore and be inspired by the natural world. Given that he was born in Nairobi and spent his early years in what is now Malawi, it seems curious that the natural abundance around him did not kindle that spark. He does speak of developing a fondness for animals, but more through reading children's books such as Hugh Lofting's *Doctor Dolittle* series, noting, "I learned late to love watching wild creatures, and I have never been such an outdoor person as either my father or my grandfather."

The African interlude came about because Dawkins's father, Clinton John Dawkins, followed family tradition by working in the British foreign service. Dawkins presents his father, who was a forester, as independent, resourceful, inventive and willing to take big risks. When a distant relative unexpectedly bequeathed to John Dawkins the country estate Over Norton Park in Oxfordshire, UK, he moved the family back to England, renounced his government pension and turned the land into a working farm. Richard, then eight, was soon packed off to boarding school.

The picture Dawkins paints of his schools is, if not quite Dickensian, pretty awful: little boys lining up naked for cold morning baths, shivering in unheated rooms, choking down bad-tasting food, enduring corporal punishment and bullying, and not always receiving a sterling education. Dawkins was, he confesses, an indifferent student, but a few teachers managed to stimulate an interest in science. It is clear that he did not come into his own intellectually until he was admitted — by the skin of his teeth — into the University of Oxford. There, at Balliol College, Dawkins was lucky enough to be tutored by the great ethologist Nikolaas 'Niko' Tinbergen, and became fascinated by animal behaviour.

➲ NATURE.COM
For a celebration of *The Selfish Gene*, see:
go.nature.com/383s7y

TERRY SMITH/TIME & LIFE PICTURES/GETTY

Richard Dawkins studying mating calls in crickets in 1976.

The latter part of the book traces Dawkins's intellectual path from Oxford to his assistant professorship in zoology at the University of California, Berkeley (where he was swept up in the movement against the Vietnam War), and his return to Oxford as a young scientist. It describes his transition from a quantitatively oriented computer-programming experimentalist who modelled chick behaviour to the more theoretically oriented scientist we know today. I doubt if more than 1 in 10,000 readers of *The Selfish Gene* ever read his earlier experimental work. (I hadn't, either.) Inspired by the 1970s ferment over group and individual selection, kin selection and the like, that book put the young Dawkins on that short list of scientists who are able to make complicated scientific ideas understandable and exciting without oversimplifying them.

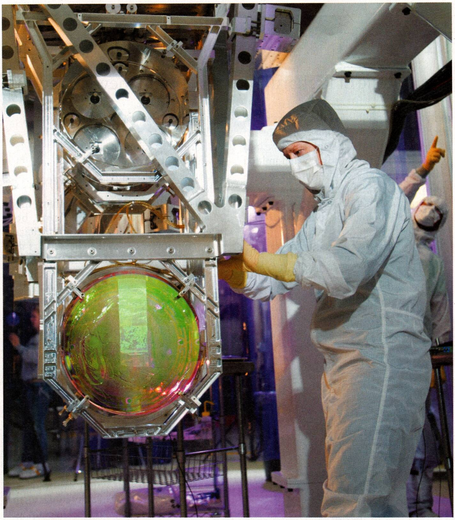
An Appetite for Wonder: The Making of a Scientist
RICHARD DAWKINS
Bantam: 2013.

Dawkins is a polarizing figure, both widely praised and widely criticized. Supporters as well as detractors may be surprised at the honest depiction of an individual who comes across as both less saintly and less diabolical than media caricatures may have led them to expect. He loves poetry, and readily confesses to choking up when reading sentimental verse such as Hilaire Belloc's 1910 *To the Balliol Men Still in Africa.* He reflects with chagrin on his lack of concern about the rampant bullying that took place at his schools, and his support of what he now considers the bullying of some Berkeley faculty members by radical professors and students during the Vietnam War protests. Of the Berkeley experience, he notes, "I was still young, but not all that young. Should have known better." He readily confesses that scientific habits were slow in coming. It is a very honest book.

Charles Darwin wrote his autobiography for his family; for whom is Dawkins's written? I found the enumeration of ancestors in early chapters a bit of a slog. The middle chapters describing his childhood in Africa and school days in England should be of interest to all. The latter chapters, dealing with his mathematical modelling of evolution, might appeal more to scientists. Dawkins's atheist fans are not all necessarily in that camp, and might find the graphs and diagrams of behaviour patterns daunting.

However, throughout and as usual, Dawkins's writing is graceful, sparkling with anecdotes and wit. Those of us who struggle in our writing will be comforted to read the words: "Pretty much every sentence I write is revised, fiddled with, re-ordered, crossed out and re-worked." In other words, the elegant, functional design of his writing is accomplished through variation and selection. Why am I not surprised? ■

Eugenie Scott *is executive director of the National Center for Science Education in Oakland, California.*
e-mail: scott@ncse.com



LIGO LABORATORY

Researchers working at the Laser Interferometer Gravitational-Wave Observatory.

SOCIOLOGY OF SCIENCE

Chasing the gravitational wave

Marianne de Laet enjoys a sociological analysis of how a select group of physicists works.

In *Gravity's Ghost and Big Dog*, British sociologist Harry Collins documents the astrophysical search for the elusive gravitational wave. In part an account of sociological fieldwork among scientists in the field and part astronomy-history mystery, Collins's book is a terrific read informed by almost 40 years of research.

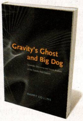
The book homes in on two sudden energy surges, thought to be the first truly significant detections of gravitational-wave signals, that got the astrophysics community in a stir: the Equinox Event in 2007 and Big Dog in 2010. The book's first part was previously published in 2010 as *Gravity's Ghost* (University of Chicago Press), a stand-alone account of the Equinox Event. The new, second part documents the frenzy over Big Dog — so named because the signal was bigger than the 2007 surge and seemed to come from the constellation Canis Major — when the stakes were higher and the future of the field hung in the balance. As an 'embedded' observer, participant, apprentice and analyst working among the astrophysicists, Collins reports on meetings, telephone conferences, e-mail discussions and social events. By conversing with and analysing scientists at research facilities, scientific conferences and home institutions, he offers a glimpse of the ways in which knowledge is made in this esoteric field.

Predicted by Albert Einstein's general theory of relativity, first published in 1915, gravitational waves are formed when the mass of an imploding star disappears, causing ripples in space time. Ground-based interferometers built to detect them have been in use for decades, but Collins focuses on the Laser Interferometer Gravitational-Wave Observatory (LIGO), which has sites at Hanford, Washington, and Livingston, Louisiana.

Two questions drive the narrative: are the signals real? And do they prove that gravitational waves exist? Collins lets us in on the conversations among the scientists. At times, they focus on the rigour of statistical margins by asking at which margin error can be ruled out; at others, they discuss the semantics of knowing, pondering whether they are confronted with observation, evidence or fact. He intersperses these exchanges with historical information and his own sociological analysis of gravitational-wave research. As the astrophysicists debate the two detection events, it emerges that deciding whether a gravitational wave exists is not just a matter of observation, calibration and learning to understand LIGO's idiosyncrasies. It also involves semantics, political considerations and attending to expectations from funders, media and the public.

A debate among key collaborators about how to announce Big Dog is a case in point. The degree of certainty with which a finding is announced sets expectations, and these find their way into the rationales for retooling or building new instruments. The validity of past research, the promise embedded in future instruments and scientific reputations are all explicitly at stake in the exchanges among the collaborators. Collins has turned the minutiae of these conversations into an exciting detective story.

Collins finds himself immersed, learning so much science that he becomes a force to be reckoned with. His queries are pertinent to the research and, by his own account, that gets the scientists to rethink strategies or interpretations of their findings. Collins even tries his hand at physics, and suggests an alternative strategy for some of the calculations for Big Dog. Flirting with the



Gravity's Ghost and Big Dog: Scientific Discovery and Social Analysis in the Twenty-first Century
HARRY COLLINS
University of Chicago Press: 2013.

science, he begins to think not only about the astrophysicists, but with them. With Collins, we begin to wonder what it takes to be an expert in the field.

In social science, going native in this way is a tricky business. In an effort not to alienate his subjects, the sociologist may end up relying too much on the group's own interpretations of their actions, leading to less insightful renderings of their world. If, by contrast, he lacks credibility with the group, his presumption to reveal something new may offend. Collins walks a tight line deftly; although his interlocutors are somewhat incredulous at his efforts to do physics, the group does engage with them.

"Collins learns so much science that he becomes a force to be reckoned with."

That does not make Collins an expert within their culture — only about it. And here, in trying to justify his calculations by asserting that they are not far off, he violates a golden rule of the sociology of science. Understanding the process of knowledge-making is not predicated on whether the knowledge in question is right or wrong. At the same time, Collins defies a best practice of anthropology — to examine one's motifs and motivations. He is not the first researcher in the sociology of science whose observation of scientific expertise turns into the desire to possess it. This interesting feature of the analysis of science remains unexamined in Collins's book.

That said, Collins's respect for science compels him to make a lovely observation: that there is something admirable about the modes of science — its aspiration to honesty, persistence and truth-seeking — and that this should serve as a moral and epistemological model for how to behave. And if, as US anthropologist Stefan Helmreich suggests in his book *Silicon Second Nature* (UC Press, 1998), social analysis of science enables participants to "recognize something new of themselves", Collins's serial monograph is on point. It tells of scientists who are well aware of their own practices — perhaps not least as a result of having a sociologist in their midst. ■

Marianne de Laet *is an associate professor of anthropology and science, technology, and society at Harvey Mudd College in Claremont, California, USA. She observed the emerging collaboration of the California Extremely Large Telescope, renamed in 2003 as the Thirty Meter Telescope.*
e-mail: delaet@g.hmc.edu

PSYCHOLOGY

Mapping memory's lanes

Alison Abbott sees the science and poetry in a penetrating study of reminiscence in the elderly.

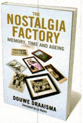
In 1688, Swiss physician Johannes Hofer coined the term nostalgia to describe the clinical symptoms of homesickness. Hofer linked the Greek words *nostos*, for homecoming, and *algia*, for pain. Nostalgia could even be life-threatening, he noted. In *The Nostalgia Factory*, psychologist Douwe Draaisma touches on this phenomenon in his exploration of memory and ageing, which draws on scientific work and anecdotal case studies.

He tells of the Dutch emigrants after the Second World War who, crippled by homesickness, later found that return offered no respite: 'home' no longer matched their memories of it. He describes the twentieth-century emigration agencies as 'nostalgia factories'. But the real nostalgia factory, he says, is time — which "makes emigrants of us all". In old age, memories are all that remain of the land of our youth, even if we never left home. His beautifully written book attempts to capture the nebulous essence of reminiscence in eight elegant, authoritative essays.

Draaisma's style is both literary and scientific. It calls to mind the works of Oliver Sacks, who popularized neuroscience through the intriguing stories of his neurological patients. (In fact, one of Draaisma's essays is based on a conversation with Sacks about "what time does to memories and what memories do to time".) But Draaisma's style is perhaps the more poetic, which is what makes the powerful insights in his book so penetrating.

It is disconcerting to learn from Draaisma how unstable and adrift our own biographies are: we constantly reconstruct our lives as our memories are refiltered through new experiences. Draaisma offers both reassurance and warnings. Forgetting names rarely foretells dementia, and many old people who describe themselves as forgetful have a well-functioning memory when tested objectively. But claims that food supplements, enriched environments or computer training programs can halt the natural process of harmless age-related forgetfulness are hokum. "Anyone who thinks that such tricks ... can actually give them a better memory probably also thinks they would be able to walk better if they used a walking frame," notes Draaisma.

For many, *The Nostalgia Factory* will be what Draaisma refers to as a 'decisive' book, one that changes one's perspective on life. Or maybe that would be true only for the young? Because in this book we also learn about the 'reminiscence-curve bump'.



The Nostalgia Factory: Memory, Time and Ageing
DOUWE DRAAISMA
Yale University Press: 2013.

Modern psychological research into reminiscences uses cue words — such as 'flag' or 'circus' — to prompt memories in elderly people, and researchers then date these to the year they happened. The number of memories recalled for each age, beginning at age three or four, follows a predictable pattern. Numbers rise to a peak at around 20 years old, then fall rapidly, flattening out at an alarmingly low level well before middle age. The curve rises again in later life, when people recall things simply because they happened recently. Middle age barely registers.

So it is not surprising that most people, if asked to name a decisive book, cite one they read before they were 23 — a time they will also describe, unprompted, as 'their era', when pop music was best. Its reflection can be seen in almost all autobiographies. In *Peeling the Onion* (Harville Secker, 2007), Nobel-prize-winning author Günter Grass devotes the majority of pages on the first 30 years of his life to those between the ages of 17 and 21.

Draaisma argues that the bump in the reminiscence curve has less to do with the ability of the young adult brain to store memories efficiently and more to do with the quality of memories accrued as we set out on independent adult life. Indeed, the bump advanced by more than a decade in a study of people who had migrated in their mid-thirties. Sacks, who emigrated from England to the United States in the 1960s at the age of 27, muses in his conversation with Draaisma that when he recorded his own year-by-year reminiscences during long driving trips, his tapes for the 1970s overflowed, but thereafter the length of the tape "decreased almost linearly".

Rigorous psychological research into the reminiscence phenomenon, whether healthy or distorted, is relatively new. This short book shows that it can reveal much about who we are at different stages of our lives. ■

Alison Abbott *is* Nature*'s senior European correspondent.*

CARDIOLOGY | ENDOCRINOLOGY | GASTROENTEROLOGY & HEPATOLOGY | NEPHROLOGY | NEUROLOGY | CLINICAL ONCOLOGY | RHEUMATOLOGY | UROLOGY

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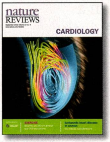

Vascular endothelial growth factor in heart failure
Z. Taimeh, J. Loughran, E. J. Birks & R. Bolli

In this Review, Taimeh *et al.* examine the evidence for the potential therapeutic application of vascular endothelial growth factor in patients with heart failure, including the novel concept of its use as an adjuvant therapy to stem cell transplantation.

www.nature.com/nrcardio

nature REVIEWS
ENDOCRINOLOGY

The endocrinology of food intake
D. P. Begg & S. C. Woods

When to eat, what to eat and how much to eat are complex processes that are influenced by many different factors. In this Review, Denovan Begg and Stephen Woods discuss how the endocrine system influences eating. The roles of satiation hormones, adiposity hormones and regulatory hormones are outlined.

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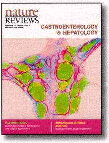

Microscopic imaging in endoscopy: endomicroscopy and endocytoscopy
M. Goetz, N. P. Malek & R. Kiesslich

Endomicroscopy and endocytoscopy not only enable prediction of histology, but actual visualization of microscopic tissue details in real time. The authors of this Review discuss these microscopic imaging devices, outlining potential indications for their use and future directions for this technology.

www.nature.com/nrgastro

nature REVIEWS
NEPHROLOGY

The primary glomerulonephritides: a systems biology approach
S. Jiang, P. Y. Chuang, Z.-H. Liu & J. C. He

The integration of omics analyses of primary glomerular diseases with data from clinical and histopathological studies is expected to advance our understanding of the molecular basis of these diseases. This article provides an overview of omics studies of primary glomerular diseases and suggests approaches to further characterize their pathogenetic mechanisms, potential biomarkers and therapeutic targets.

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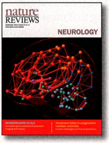

Treatment trials in progressive MS— current challenges and future directions
M. W. Koch, G. Cutter, P. K. Stys, V. W. Yong & L. M. Metz

Koch and colleagues describe current limitations of trials in progressive multiple sclerosis, such as lack of suitable outcome measures, and present approaches to address these challenges.

www.nature.com/nrneurol

nature REVIEWS
CLINICAL ONCOLOGY

Luminal breast cancer: from biology to treatment
M. Ignatiadis & C. Sotiriou

Luminal breast cancer is the most-frequently occurring breast cancer subtype. Data for the stratification of patients to the optimal treatment are emerging. This Review outlines the biology of this disease, and discusses the treatment options and ongoing studies for women with oestrogen receptor-positive breast cancer.

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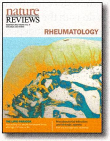

Bedfellows: mycobacteria and rheumatoid arthritis in the era of biologic therapy
K. L. Winthrop & M. Iseman

Epidemiological data and mechanistic explanations for the increased risk of mycobacterial infection in patients with RA are reviewed herein, and appropriate strategies to prevent and manage these comorbidities are outlined.

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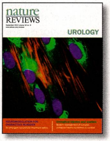

Management of combat-related urological trauma in the modern era
M. Williams & J. Jezior

Complex genitourinary injuries have emerged as common occurrences in current military combat operations. In this Review, the authors examine the current management strategies for combat-related injuries with specific focus on the lower urinary tract and genitalia.

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nature REVIEWS

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Correspondence

Turkey's scientists can't ignore politics

Given the severity of the political problems that scientists face in Turkey, academics need to become more politically involved — not less, as you suggest — to help bring about reform to the country's academic system (see *Nature* **500,** 253; 2013).

There is no clear evidence to support your implication that the debate over the headscarf ban in Turkish universities has affected the quality of their scientific research. Other long-standing impediments are the real culprits.

Scientists in Turkey have had to contend for years with a lack of academic freedom and transparency in grant-review and faculty-recruitment processes. The small size of the academic community and a clumsy bureaucracy further obstruct the nation's research potential.

There are some notable successes, however. Contrary to your description of Boğaziçi University as "cash-starved", in 2010 researchers there gained funding of close to US$13 million, of which more than $2 million came from highly competitive international sources (see go.nature.com/wik8xn).

Ozan Aygün *Massachusetts Institute of Technology, Cambridge, USA.*
oaygun@mit.edu

Transcranial devices are not playthings

Controlled investigation of transcranial direct-current stimulation (tDCS) for treating neuropsychiatric disorders or for neurorehabilitation should not be confused with improvised devices or practices that apply electricity to the brain without reference to established protocols (see *Nature* **498,** 271–272; 2013). Unorthodox technologies and applications must not be allowed to distort the long-term validation of tDCS.

Experimentation outside established and tested norms may put subjects at risk. In tDCS, the delivered dose of electrical brain stimulation (defined by the waveform and intensity applied) and the electrode size, number and position are all crucial. Safe and effective dose ranges have been established in clinical trials. Patients receiving tDCS do so in a controlled environment, under guidance from institutional ethics review boards and with strict criteria for patient inclusion.

Meddling with the tDCS dose is potentially as dangerous as tampering with a drug's chemical composition. Painstaking efforts by researchers to understand the risks and benefits of tDCS should never be interpreted as encouraging such practices.

Marom Bikson *City College of New York, USA.*
bikson@ccny.cuny.edu
Sven Bestmann *University College London, UK.*
Dylan Edwards *Burke–Cornell Medical Research Institute, New York, USA.*
M. B. declares competing financial interests: see go.nature.com/lxuxfq for details.

Don't compromise on informed consent

I urge researchers responding to the call for information about sharing data sets (M. Bobrow *Nature* **500,** 123; 2013) to defend the important principle of informed consent by patients participating in medical research.

The UK Health and Social Care Act 2012 requires the transfer of all National Health Service electronic medical records in England from general practitioners to the Health and Social Care Information Centre. The biomedical research charity the Wellcome Trust and the Human Genomics Strategy Group have proposed that variant files, which contain whole human genomes minus the Sanger Institute reference genome, should be attached to the records.

The UK government's intention is to share these data with accredited researchers — ranging from the Google-funded gene-testing company 23andMe to private health-care companies and Chinese research institutes — but without people's knowledge or consent.

Most data will be 'pseudo-anonymized', with some identifiers (such as names) stripped out, but with the ability to link back to the individual retained. People can opt out of this data-sharing system (see go.nature.com/azkyru), but that will in effect prevent members of the public from taking part in medical research.

Abandoning informed consent is unlikely to benefit biomedical research (J. P. A. Ioannidis *Am. J. Bioethics* **13,** 40–42; 2013). Researchers could face a dwindling source of data as people withdraw from participation to protect their confidentiality.

Helen Wallace *GeneWatch UK, Buxton, UK.*
helen.wallace@genewatch.org

HeLa genome versus donor's genome

I contend that the continual divergence of chromosomal features ('karyotype') and DNA sequence in dynamic cancer-cell populations undermines debate over ownership of the HeLa cancer-cell line derived from Henrietta Lacks six decades ago (see *Nature* **500,** 121 and 132–133; 2013).

The HeLa genome is no longer Henrietta Lacks's personal genome. Although the two share some DNA sequences, the similarity ends there. Lacks's genome had the usual number of 46 normal chromosomes, whereas most HeLa cells have 70–90 chromosomes and more than 20 translocations, some of which are highly complex.

Changes in the HeLa genome in the past few decades have resulted from multiple cycles of genome reorganization during the cancer process and from the initial cell-culture experiments. Considering that chromosomes provide the genome identity and blueprint, it might even be argued that the HeLa genome is no longer a human genome.

Henry H. Heng *Wayne State University School of Medicine, Detroit, USA.*
hheng@med.wayne.edu

A forgotten history of sex research

Elizabeth Pollitzer's point about sex mattering in all areas of biology has long been considered an important topic (see *Nature* **500,** 23–24; 2013).

For example, the Endocrine Society's flagship journal *Endocrinology*, of which I am editor-in-chief, has since 2012 required authors to include the sex of animals, tissues and even cell lines in their papers (J. D. Blaustein *Endocrinology* **153,** 2539–2540; 2012). Authors must justify the use of single-sex animals or tissues, and consider sex in interpreting their data.

Research in neurobiology and psychology has also consistently recognized sex differences in physiology, pathophysiology, pharmacology and toxicology (see, for example, G. E. Gillies & S. McArthur *Pharmacol. Rev.* **62,** 155–198; 2010). The US National Institutes of Health supports such research and has emphasized its importance in programme announcements.

From 1922, the US Committee for Research in Problems of Sex was funded by the Rockefeller Foundation for decades. In 2006, the Organization for the Study of Sex Differences, based in Atlanta, Georgia, was founded for researchers and clinicians.

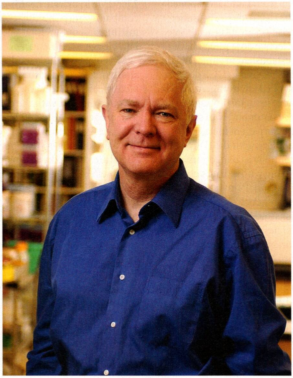
Studies on sex differences published before the PubMed online archive, electronic journals and searchable keywords should not be overlooked.

Andrea C. Gore *University of Texas at Austin, USA.*
andrea.gore@austin.utexas.edu

Anthony James Pawson

(1952–2013)

Biochemist whose vision of cell signalling transformed cancer research.



UNIV. TORONTO

Tony Pawson's research on protein interactions transformed the thinking about how cells communicate, how proteins evolve and how cellular messaging goes awry in cancer. A creative experimenter, his synthesis of diverse observations in areas from biochemistry to mouse genetics and developmental biology led to a coherent picture of how cellular processes work.

In the 1980s, early in his career, Pawson and his team discovered the Src homology region 2 (SH2). A subunit, or domain, of many proteins, SH2 directs how proteins interact and governs how cells respond to external cues. This finding set a path for all his future work.

Pawson went on to show that combinations of a small number of domains could produce an enormous range of cellular responses. This 'modular' vision reshaped scientists' understanding of cellular regulation and paved the way for the development of drug classes that interfere with these protein interactions.

But recognition did not come immediately for Pawson. The existence of modular binding domains, now standard textbook fare, was initially received with scepticism by biochemists, and with benign neglect by molecular biologists. But as the evidence, largely from Pawson's lab, grew more compelling, it could no longer be ignored.

Pawson, who died suddenly at home on 7 August aged 60, was born in Maidstone, UK, to an eminent British family. His father, to some the more famous Tony Pawson, was a champion sportsman and a household name in Britain. A fly fisher, cricketer, footballer and, later, sports writer, his multivalent skills loomed large for the younger Pawson, who was often mistaken for his father. It was his mother, Hilarie, a biology teacher, who stimulated his interest in science.

Pawson read biochemistry at the University of Cambridge, UK, and obtained his PhD in 1976, working with Alan Smith at the Imperial Cancer Research Fund (now Cancer Research UK) on proteins encoded by the Rous sarcoma retrovirus. While visiting a friend in Cambridge, Tony met his American wife-to-be, Maggie. They married in 1975, and in 1976 moved to Berkeley, California, where Tony began postdoctoral work on the protein products of avian retroviruses.

In 1981, the couple moved to Vancouver, Canada, where Pawson was assistant professor in the department of microbiology at the University of British Columbia. Pawson's lab became immediately productive, publishing important papers on oncoproteins — proteins coded by genes that have the potential to cause cancer. There, Pawson struck up collaboration with Mike Smith, a Nobel-prizewinning chemist who invented the technique of site-specific mutagenesis that Pawson used in his SH2 discovery.

In 1985, when a research institute was launched at the Mount Sinai Hospital in Toronto (now the Lunenfeld–Tanenbaum Research Institute), Pawson joined us as one of the first appointments in its molecular and developmental biology division. With the addition of developmental biologist Alexandra Joyner and the late molecular biologist Martin Breitman, the five of us — young, ambitious and with a pioneering spirit — knew that we were building something important.

The division was created at a propitious time: developmental biology was about to be transformed by the latest genetic technologies from a descriptive to a mechanistic science, and cancer research was accelerating with the discovery of oncogenes, tumour-suppressor genes and related signalling pathways. The two fields were about to converge with the discovery that the normal equivalents of viral oncogenes have crucial roles in embryo development.

Within a few years, the division grew from having just a handful of students and postdocs to having more than 100 members. Pawson was at the centre, partly because cell signalling was core to all our science, but largely because he loved to collaborate. To him, collaboration was as much about camaraderie and friendship as it was about getting a piece of science done. Working with Tony was fun, and although he was invited to give ten times the number of talks as everybody else, he would always give credit to his collaborators.

Pawson's seminars were virtuoso performances, and they were eagerly attended. His talks and more than 450 published papers were not just assemblages of data, but elegantly presented expositions of how cells and organisms evolve, develop and function. At the time of his death, he was one of the most highly cited biomedical researchers.

When Pawson received the Heineken Prize for biochemistry and biophysics in 1998, he spoke at the ceremony in Amsterdam about the joy of discovery, the privilege of working with talented young people, the potential for advances to lead to new treatments for disease, and about the importance of family in a scientist's life. One could have heard a pin drop.

Our strongest memories of Tony are in those early years at the Lunenfeld, sharing our latest results, writing grants, exchanging gossip and sharing family joys and sorrows — and watching Tony gesticulating wildly with his arms when he got excited. His enthusiasm was infectious. He will be greatly missed. ■

Alan Bernstein *is president and chief executive officer of the Canadian Institute for Advanced Research in Toronto.*
Janet Rossant *is chief of research at the Hospital for Sick Children in Toronto.*
e-mails: abernstein@cifar.ca; janet.rossant@sickkids.ca

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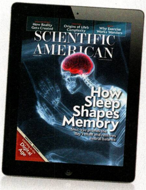
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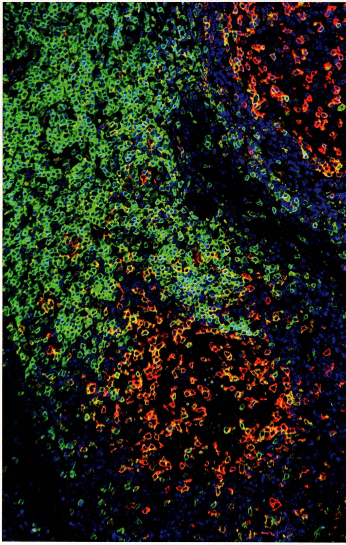

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RESEARCH

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Papers published this week at Nature.com/nature

NEWS & VIEWS Statistical evidence that firearms restrictions *do* reduce the muder rate **p.170**

ARTICLE While the cat's away ... the mice will be high on oxytocin and 5HT **p.179**

LETTER Millions across Asia are exposed to arsenic-tainted drinking water **p.204**

PUBLISHED 8 SEPTEMBER 2013

GENOMIC INSTABILITY

Two replication fork maintenance pathways fuse inverted repeats to rearrange chromosomes

L Hu *et al.*
doi:10.1038/nature12500

EPIGENETICS

Uhrf1-dependent H3K23 ubiquitylation couples maintenance DNA methylation and replication

A Nishiyama *et al.*
doi:10.1038/nature12488

CELL BIOLOGY

Cyclin A regulates kinetochore microtubules to promote faithful chromosome segregation

L Kabeche & D A Compton
doi:10.1038/nature12507

PUBLISHED 11 SEPTEMBER 2013

PHOTOCHEMISTRY

Solar cells made simpler

Nanostructured solid-state solar cells that incorporate organometallic halide perovskites as the light-absorbing material have recently attained levels of power-conversion efficiency that are attractive for practical applications. Now, Mingzhen Liu *et al.* show that, when using vapour deposition rather than the usual solution processing to deposit the perovskite, high efficiencies can be achieved with these materials in even simpler device structures. By removing the need for complex nanostructures altogether, the prospects for large-scale manufacture are potentially enhanced.

Efficient planar heterojunction perovskite solar cells by vapour deposition

M Liu *et al.*
doi:10.1038/nature12509

Fast-track solar cells

M D McGehee
doi:10.1038/nature12557

IMMUNOLOGY

Vaccine combats virulent SIV

Work on potential vaccines against HIV and the simian equivalent (SIV) has so far proved largely fruitless. This study makes some progress by exploiting the recent observation that the pathogens seem to be vulnerable to immune control or pharmacological clearance in the first few hours to days of infection. Rhesus macaques vaccinated with SIV protein-expressing rhesus cytomegalovirus (RhCMV/ SIV) vectors developed durable resistance to the highly pathogenic SIVmac239 after challenge by vaginal and intravenous routes. Some of the vaccinated animals have controlled viral replication for 1–3 years with no demonstrable evidence for residual virus, raising the possibility that the vaccine-elicited immune responses may, in fact, have cleared the initial infection.

Immune clearance of highly pathogenic SIV infection

S G Hansen *et al.*
doi:10.1038/nature12519

STEM CELLS

In vivo production of iPS cells

Manuel Serrano and colleagues show for the first time that reprogramming of somatic cells to pluripotency by the classic 'Yamanaka factors' *Oct4*, *Sox2*, *Klf4* and *cMyc* can be achieved *in vivo*. Analysis of induced pluripotent stem (iPS) cells induced *in vivo* from stomach, intestine, pancreas and kidney cells in mice shows that they are closer to embryonic stem cells than are *in vitro*-generated iPS cells. The *in vivo* iPS cells also have the potential to generate embryo-like structures that express embryonic and extraembryonic markers, which suggests that they have totipotent features not found in conventional iPS or embryonic stem cells.

Reprogramming *in vivo* produces teratomas and iPS cells with totipotency features

M Abad *et al.*
doi:10.1038/nature12586

Reprogramming *in situ*

A De Los Angeles & G Q Daley
doi:10.1038/nature12559

Usp16 contributes to somatic stem cell defects in Down's syndrome

M Adorno *et al.*
doi:10.1038/nature12530

Down's syndrome link to ageing

G P Souroullas & N E Sharpless
doi:10.1038/nature12558

PLANT BIOLOGY

A novel GPCR in maize

In plants the shoot apical meristem stem cells, which are responsible for stem growth, are maintained by a negative feedback loop between the CLAVATA signalling pathway and the *WUSCHEL* homeobox gene. In this study, David Jackson and colleagues demonstrate that in maize, the *COMPACT PLANT2* (*CT2*) gene encodes a Gα subunit of a heterotrimeric GTP binding protein. CT2/Gα transmits a stem cell restrictive signal from a maize CLAVATA leucine-rich repeat receptor-like protein, FEA2, suggesting a previously unrecognized function for Gα signalling in plants. Single-pass transmembrane receptors act as G-protein coupled receptors (GPCRs) in plants, challenging the widely held assumption that GPCRs are exclusively seven-transmembrane domain receptor proteins

The maize Gα gene *COMPACT PLANT2* functions in CLAVATA signalling to control shoot meristem size

P Bommert *et al.*
doi:10.1038/nature12583

GENETICS

Migrating bubble during break-induced replication drives conservative DNA synthesis

N Saini *et al.*
doi:10.1038/nature12584

Pif1 helicase and Polδ promote recombination-coupled DNA synthesis via bubble migration

M A Wilson *et al.*
doi:10.1038/nature12585

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NEWS & VIEWS

SOCIAL SCIENCE

The mathematics of murder

A mathematical model of gun ownership has been developed that clarifies the debate on gun control and tentatively suggests that firearms restrictions may reduce the homicide rate.

ADELINE LO & JAMES H. FOWLER

The mass killing of 20 children and 6 adults on 14 December 2012 at Sandy Hook Elementary School in Newtown, Connecticut (Fig. 1), has revived an enduring controversy about gun control in the United States. Gun-control advocates believe that widespread gun ownership increases the rate of gun-related crime and homicide, whereas critics argue that gun availability actually decreases gun violence because potential assailants are less likely to commit such crimes if they believe citizens are armed. But who is right? In an article published in *PLoS ONE*, Wodarz and Komarova[1] describe an elegant and highly parsimonious mathematical model designed to answer exactly this question. And, in an extremely cautious way, they suggest that more guns make things worse.

The scientific literature suggests that gun homicides are influenced by various interwoven factors, including the rate of legal and illegal gun possession, the national prevalence of armed and unarmed attacks, the likelihood of fatalities in such attacks, and the quality and quantity of general law enforcement[2–5]. Gun-control policy is clearly only one key variable in a complex social system. But work on the issue is typically conducted by scholars who collect data on types of gun-control policy and numbers of gun homicides, and look for correlations while controlling for certain important variables — such as whether the locations of the homicides are rural or urban, or whether it is easy to obtain arms illegally.

The problem is that many of these correlations are difficult to interpret. If gun deaths are higher in states with stricter gun-control laws, is this because gun restrictions cause higher crime or because politicians react to higher crime by enacting more restrictions? It is with this difficulty in mind that Wodarz and Komarova have created their formal model of gun ownership.

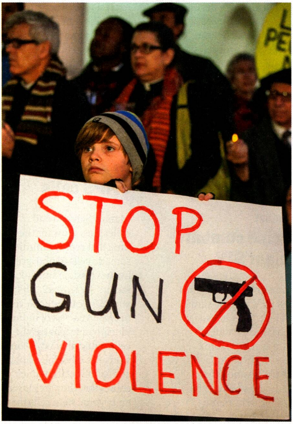

Figure 1 | A prayer vigil for the victims of the Sandy Hook shooting.

DAMIAN DOVARGANES/AP/PRESS ASSOCIATION IMAGES

The modelling of complex social phenomena is not new. As early as 1974, the economist Gary Becker took a supply-and-demand approach to the 'production' of crime and punishment, using his model to show how crime might be minimized with various public and private policies that make criminal behaviours 'costly'[6]. But Becker's analysis did not explicitly tackle the thorny problem of gun control, and the scholars who followed also tended to focus more on abstract models.

By contrast, Wodarz and Komarova's model is explicitly designed to address gun-control policies and their effects. At the core of the model is the rate of gun ownership. Strict laws might lower the rate and permissive laws might increase it, but the mechanism itself does not matter — the goal of the model is to clarify what assumptions are necessary to measure the effect of overall gun ownership on the rate of firearms-related homicides.

Wodarz and Komarova assume that there is a positive relationship between the number of gun owners and the number of potential gun-related attackers. This is reasonable: if there are no guns, there will be no attacks with guns. But the authors also assume that there is a negative relationship between the rate of gun ownership and the likelihood that a gun-wielding attacker actually uses his or her weapon. This is because non-criminals may own guns, too. If a potential victim possesses a gun, a potential attacker might think twice about attacking.

A few other factors are also included in the model, such as the risk of dying in a gun attack, and the availability and take-up of illegal arms in the face of varying levels of gun control. But the key insight is that there are essentially two perfect worlds, one in which no one owns a gun (meaning no one is able to attack) and one in which everyone owns a gun (meaning no one is willing to attack). In between, we get the worst of both worlds because some criminals have guns and they choose to use them. This means that the effect of gun availability is crucially dependent on where we sit between these two worlds.

This is social science at its very best. Rather than crafting yet another highly abstract formal model, Wodarz and Komarova create a model that is directly relevant to an extremely important societal issue. And rather than overly emphasizing the results of their model, they conduct an exercise in caution, highlighting the importance of grounding models in sound and accurate assumptions — because a model is only as good as the assumptions from which it proceeds.

Using their best guess about values estimated or implied from the existing literature, Wodarz and Komarova show that their model implies that stricter laws are the best way to reduce gun deaths. But they are quick to point out that there are some assumptions that are vital to the model for which we have no reliable measurement. So although the model does lend support to arguments in favour of gun control, it is fair to say that the jury is still out.

The most obvious contribution Wodarz and Komarova make towards the study of gun-control policies is in highlighting key parameters that require further empirical investigation. Collaborative efforts between sociologists, political scientists and other scholars can now move forward in an objective way to advance our understanding of gun-control policies by focusing on the assumed values that are currently less well measured. Armed with these, the model might help us to resolve — perhaps once and for all — the debate on gun ownership. ■

Adeline Lo *and* **James H. Fowler** *are in the Political Science Department, University of California, San Diego, La Jolla, California 92093-0521, USA.*
e-mail: fowler@ucsd.edu

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BIOLOGICAL TECHNIQUES

An embryonic view of tumour development

A genome-wide screen of developing mouse embryos, performed using RNA-interference techniques, finds new suspects in skin cancer. But some factors seem to have opposing roles in cancer and normal-tissue maintenance. SEE ARTICLE P.185

PAWEL J. SCHWEIGER & KIM B. JENSEN

Gene function has traditionally been studied using organisms in which individual genes have been either eliminated or artificially expressed in excess. The discovery in the 1990s of RNA interference (RNAi) — the process by which small RNA molecules specifically inhibit gene expression by binding to and destroying messenger RNAs — established a new framework for investigating gene function. Until now, however, genome-wide screens using RNAi techniques have been limited mainly to cultured cells, and so cannot take into account the complex cellular interactions that govern normal tissue behaviour or that lead to the development of diseases such as cancer. On page 185 of this issue, Beronja *et al.*[1] describe the first *in vivo* genome-wide RNAi screen, which was performed in mouse embryos with the aim of identifying genes involved in the development of non-melanoma skin cancer*.

Non-melanoma skin cancer — one of the most prevalent tumour types — occurs in the epidermal cells that make up the outer layer of the skin. In the developing embryo, the epidermis forms during mid-gestation from the surface ectoderm and gradually matures into an impermeable barrier with associated structures, such as hair follicles. Early studies of non-melanoma skin cancer identified members of the *Ras* gene family as potent drivers of the disease[2], but a lack of techniques for studying gene activity at the single-cell level[3] has meant that little is known about other genes involved in the maintenance of these, and other, tumours.

The team presenting the current paper previously described an approach[4] for achieving RNAi in the developing epidermis. In this technique, cells in the surface ectoderm of mouse embryos are transduced by means of lentiviral vectors carrying short hairpin RNAs (shRNAs), which are cleaved to RNAi sequences once the shRNAs are expressed in a cell. The researchers have now extended this approach to screen the entire genome of the epidermal cells, using a library of shRNA molecules designed to interfere with every mRNA in mouse cells. Because cells in the developing epidermis divide in an extremely reproducible manner, any RNAi sequence that alters the normal rate of proliferation can be identified as either enriched or depleted in a pool of RNAi sequences. Thus, by comparing the relative abundance of shRNA sequences in the embryos' genomic DNA at the time of RNAi induction (embryonic day 9.5) and nine days later, the authors were able to identify genes that, when inhibited, cause proliferative advantages or disadvantages.

Beronja and colleagues conducted two such screens: one to assess genes involved in normal epidermal development and the other to assess genes implicated in the development of tumours induced by enhanced expression of *Hras1*. The top candidate gene to emerge in both screens, but with an opposite effect on proliferation, was that encoding the β-catenin protein. This finding is in line with previous reports[5,6] showing that β-catenin is required for the development of non-melanoma skin cancer and that loss of this protein specifically in the epidermis causes hyperproliferation.

β-Catenin is an integral component of adherens junctions — protein complexes that form between cells in epithelial and endothelial tissues — and is therefore key to

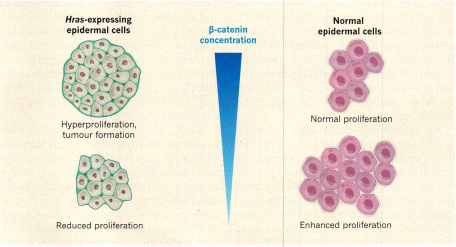

Figure 1 | Role of β-catenin in normal and cancer-prone skin cells. Beronja and colleagues' *in vivo* whole-genome RNA interference (RNAi) screen[1] of epidermal cells expressing the oncogene *Hras* identified β-catenin as essential for maintaining cellular hyperproliferation in this model of cancer; inhibiting expression of the protein led to decreased proliferation. The authors infer that this effect is mediated through β-catenin's involvement in the Wnt signalling pathway. By contrast, the same screen in normal epidermal cells revealed that RNAi-induced inhibition of β-catenin led to enhanced proliferation in such cells. Follow-up experiments suggest that this effect results from β-catenin's role in maintaining the adherens-junction protein complexes between epidermal cells; disruption of these junctions impairs the contact-inhibition processes that keep cell proliferation in check.

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*This article and the paper under discussion[1] were published online on 14 August 2013.

intercellular adhesion. It is also a cofactor of the Lef1/Tcf family of transcription factors, which are effector molecules in signalling pathways involving Wnt proteins[7]. β-Catenin thereby regulates several fundamental cellular processes. In the authors' screen for regulators of normal epidermal-cell behaviour, β-catenin emerges as a negative regulator of epidermal-cell growth (Fig. 1). Follow-up *in vitro* experiments suggested that this effect is independent of Wnt signalling, and that it stems from a loss of contact-mediated inhibition of proliferation. By contrast, the authors show that β-catenin is required for *Hras1*-induced hyperproliferation, and that this correlates with increased Wnt signalling *in vivo*. Intriguingly, however, a previous study showed[8] that blocking Wnt signalling in the epidermis by expression of an inhibitory form of Lef1 does not impede epidermal tumour formation. Future work is therefore needed to evaluate the involvement of β-catenin and Wnt signalling in tumour development.

Whereas β-catenin has already been implicated in numerous malignancies, Beronja and colleagues' screen identified another gene, *Mllt6*, as also being involved in *Hras1*-induced epidermal-cell hyperproliferation. Chromosomal translocations of this gene have previously been associated with mixed myeloid leukaemia[9]. Although the exact function of the Mllt6 protein is unclear, it is known to influence the subcellular localization of the Dot1a–histone methyltransferase protein complex and to regulate specific gene networks[10]. Like β-catenin, Mllt6 is required for tumour growth induced by *Ras*-gene mutations. The existence of multiple Lef1/Tcf binding sites in the *Mllt6* promoter indicates that it is a potential downstream target of β-catenin-mediated Wnt signalling. However, further investigations are required to determine whether Mllt6 is directly regulated by β-catenin, whether concerted action of the two proteins drives hyperproliferation, or whether Mllt6 is an essential component of another signalling pathway. This will be important to resolve, because it influences the number of potential downstream drug targets.

The bird's-eye perspective taken by Beronja *et al.* to identify regulators of growth has produced a wealth of information. The extensive data sets generated in their study provide the basis for further comprehensive computational analysis. A subset of the genes identified is probably involved in epidermal-appendage formation, rather than strictly in proliferation. Filtering the data on the basis of gene-expression patterns during this phase of embryo development would provide an excellent framework for categorizing genes into those with functions in homeostasis, development and oncogene-induced growth. Moreover, it will be exciting to see whether the genes implicated in Ras-protein-induced growth represent universal mechanisms for abnormal proliferation, or if different tissues have evolved discrete mechanisms for controlling homeostasis. ■

Pawel J. Schweiger *and* **Kim B. Jensen** *are at the Biotech Research and Innovation Center, University of Copenhagen, DK-2200 Copenhagen N, Denmark.* **K.B.J.** *is also at the Wellcome Trust and Medical Research Council Cambridge Stem Cell Institute, Cambridge, UK.*
e-mail: kim.jensen@bric.ku.dk

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MATERIALS SCIENCE

Boosting X-ray emission

A spectroscopic technique has been demonstrated that uses stimulated emission to enhance weak X-ray signals for fundamental studies in materials science. SEE LETTER P.191

ERNST FILL

During the past decade or so, a spectroscopic technique called resonant inelastic X-ray scattering (RIXS) has evolved as a powerful tool for probing elementary excitations in materials[1,2]. Excitations accessible to RIXS include charge transfer, crystal-field excitations, magnons, molecular vibrations and even phonons. Recent progress in this field is due to the availability of third-generation synchrotrons as X-ray sources and to advances in X-ray photon detection[2]. However, the method still suffers from a low photon yield because of dominant radiationless processes, requiring high incident photon fluxes that cause damage to the sample under investigation. On page 191 of this issue, Beye *et al.*[3] demonstrate an ingenious way to greatly increase the photon yield for RIXS.

In RIXS, a beam of X-ray photons is directed at a sample, and the photon energy is chosen such that photon absorption elevates an electron from a deep-lying atomic-core energy level to a previously unoccupied state in the material's valence energy band. This process generates a hole — a particle created by the absence of an electron — in the atomic core. This highly unstable state typically decays within a few femtoseconds (1 fs is 10^{-15} s; in the current experiment the core-hole lifetime is 19 fs). Usually, the main decay channel is the Auger process, in which an electron jumps to the core hole and another electron is emitted. RIXS relies on a different decay process in which, again, an electron jumps to the core but, instead of an electron, a photon is emitted

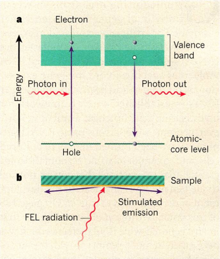

Figure 1 | RIXS spectroscopy using stimulated emission. a, In conventional RIXS, an incident photon generates a hole in a deep-lying atomic-core energy level and an electron in the unoccupied part of the valence energy band (left). An electron from the occupied part of the valence band fills the core hole, leaving a hole in the valence band and emitting a photon (right). The emitted photon provides information about energy states of the underlying material. **b,** In Beye and colleagues' experiment[3], the downward transition is amplified by stimulated emission. A brilliant soft-X-ray free-electron laser (FEL) beam is focused on a crystalline silicon sample and penetrates only about 1 micrometre into the sample (yellow). A high percentage of atoms is excited, allowing stimulated emission to predominate over spontaneous emission and other decay channels. This results in a highly directional emission close to the surface.

(Fig. 1a). The fluorescence yield (the ratio of the rate of radiative decay to total decay rate) is very low, and thus the RIXS signal is weak. Nevertheless, RIXS provides a large amount of information on the chemical and physical features of materials.

In their study, Beye *et al.* irradiated a solid-state sample with a soft-X-ray free-electron laser (FEL), which allows a large fraction of atoms to be excited. In this way, a mechanism known as stimulated emission takes over and the radiative channel is significantly enhanced at the expense of non-radiative processes; in stimulated emission, an incident photon de-excites an excited state by generating a second photon with exactly the same energy and direction. FEL radiation had previously been proposed for excitation[2]. FELs emit brilliant, tunable, monoenergetic photon beams that can be focused to spot sizes smaller than 10 micrometres, inducing a high density of photons (intensity) on the sample. But Beye and colleagues went one step further and applied FEL radiation to generate the sizeable atomic population inversion that is required to induce X-ray amplification (gain) and generate laser light at the energies of the transitions investigated.

The method is related to the concept of an inner-shell X-ray laser. This laser scheme had already been suggested in the 1960s[4], but has only quite recently been realized, also using FEL radiation[5]. In the current experiment, the gain generated is so high that the laser transition becomes saturated — that is, almost all of the excited states are converted into photons and the non-radiative channel is effectively quenched. The emitted radiation is highly directional and radiated close to the sample surface (Fig. 1b). Directionality and amplification may lead to an enhancement of the brilliance of the emitted photon beam by several orders of magnitude, solving the problem of the weak RIXS signal.

In Beye and colleagues' pioneering experiment, the conditions for observing stimulated emission were not optimal. Owing to experimental constraints, the spectroscopic observation had to be limited to a detection angle to the surface of 15°, whereas the emission maximum was found to occur at 9°. Because of this problem, an enhancement of only a bit more than a factor of two was measured. However, the high directionality of the beam was demonstrated clearly, showing the effect of stimulated emission.

The authors' experiment is an important first step towards a new way of using RIXS for fundamental studies. Possible improvements include an elongation of the irradiated spot for increasing the directionality; travelling-wave excitation (in which the excitation sweeps over the sample at the speed of light, with the generated radiation exactly following the excitation) to avoid decay of population inversion before the beam has left the excited material; and a better match of the observation direction to the emission direction.

An open question is how the signal in various spectral regions might be altered by the stimulated-emission process. If all transitions are ensured to be saturated, this may not be a problem. Another challenge pertains to the high directionality of the emission: how can the information on the excitations, which depends on the change in momentum that the photons undergo, be retrieved under this condition? In ordinary RIXS, photons are emitted in different directions and such information is derived from this feature.

Another point concerns the incident-photon energy at which stimulated-emission RIXS can be carried out. With a photon energy of around 100 electronvolts, Beye and colleagues' experiment falls into the very-low-energy regime of RIXS. Higher photon energies are already available with FELs — for example, at the Linac Coherent Light Source at the SLAC National Accelerator Laboratory in Menlo Park, California, which emits photons with energies of up to 10 keV (ref. 6). With more-energetic photons, stimulated-emission RIXS may revolutionize investigations of many chemical elements. However, the shorter lifetime of the excited states that are reached at such photon energies will make inducing stimulated emission more challenging. Application of the technique may be extended to liquids or even gases in order to investigate free atoms or molecules[7]. Owing to the method's increased spectral resolution, the investigation of new low-energy excitations (for example, phonons with energies much below 0.1 eV) may be possible.

This work could open up a new chapter in RIXS studies and lead to the discovery of novel excitations. A further exciting prospect is the potential to make time-resolved measurements using two incident photon beams[8]. ■

Ernst Fill *is at the Max Planck Institute of Quantum Optics, D 85748 Garching, Germany.*
e-mail: ernst.fill@mpq.mpg.de

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IMMUNOLOGY

Lipopolysaccharide sensing on the inside

Host-cell detection of lipopolysaccharide in the outer membrane of Gram-negative bacteria was thought to be restricted to the cell-surface receptor TLR4. It emerges that lipopolysaccharide can also be sensed in the cytoplasm.

VIJAY A. K. RATHINAM
& KATHERINE A. FITZGERALD

The innate immune system provides an exquisite defence system against microorganisms, but its excessive engagement can be dangerous. Overproduction of cell-signalling molecules called cytokines and excessive release of other components of dying cells can directly or indirectly injure host tissues and, in extreme cases, may lead to blood poisoning, or sepsis. The primary trigger of such reactions is lipopolysaccharide, the main component of the outer membrane of Gram-negative bacteria. Genetic studies suggested that this component is sensed exclusively through Toll-like receptor 4 (TLR4), a member of an ancient receptor family dedicated to the detection of infectious microorganisms[1,2], but there were some hints of TLR4-independent sensing of lipopolysaccharide[3].

In an exciting paper published in *Science*, Kayagaki *et al.*[4] describe a TLR4-independent sensing mechanism for lipopolysaccharide (LPS) that occurs in the cytoplasm of macrophages — a type of innate immune cell*. Although the identity of the novel LPS receptor remains unknown, the authors show that its engagement results in activation of the inflammatory enzyme caspase-11. The findings greatly enhance our understanding of LPS responses and may have implications for the treatment of sepsis.

The discovery emerged from the authors' examination of inflammasome activation during Gram-negative bacterial infections. Inflammasomes are large, multi-protein complexes found in the cytoplasm that couple pathogen recognition to the maturation of IL-1β and other cytokines. Inflammasomes

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*This News & Views article was published online on 4 September 2013.

also trigger an inflammatory cell-death response called pyroptosis. The best-studied inflammasome contains the protein NLRP3, a sensor of microbial infection that engages the adapter molecule ASC, which then recruits and activates the effector enzyme caspase-1. Although most activators of NLRP3 lead to caspase-1 engagement by this mechanism, previous work[5] from the research group presenting the current paper showed that, during infections with gut pathogens such as *Escherichia coli* and *Vibrio cholerae*, engagement of caspase-11 was essential to facilitate NLRP3–ASC-dependent caspase-1 activation and IL-1β maturation (Fig. 1). The caspase-11 pathway was also shown to play a part in LPS-induced septic shock.

Several other studies[6–13] subsequently linked caspase-11 to inflammasome responses for a broad range of Gram-negative (but not Gram-positive) bacterial pathogens, and attempted to understand how caspase-11 is activated in these infections. Kayagaki and colleagues now show that cytoplasmic LPS recognition has a role.

The findings came by a circuitous route. The group's previous work[5] had identified cholera toxin B (CTB) as a trigger of the caspase-11 pathway in cells exposed to LPS. The latest work describes how one form of LPS, O111:B4, facilitates caspase-11 activation by CTB, whereas other LPS serotypes or lipid A — the portion of LPS that is responsible for TLR4 activation — do not. It turns out that CTB is not itself a trigger of caspase-11 activation but instead acts as a vehicle to deliver O111:B4 LPS into the cell. This finding led the authors to speculate that cytoplasmic LPS is the trigger that activates caspase-11, and they validated this idea by showing that LPS and lipid A led to caspase-11 activation when they were delivered directly to the cytoplasm.

Following experiments showing that a modified form of LPS that still activates TLR4 failed to trigger the cytoplasmic LPS-sensing pathway, Kayagaki *et al.* hypothesized that cytoplasmic LPS is sensed in a TLR4-independent manner. In support of this idea, they show that TLR4-deficient macrophages undergo normal caspase-11-pathway activation in response to LPS or lipid A, as long as the cells are first primed with ligands of other Toll-like receptors, TLR2 or TLR3, to induce expression of NLRP3, IL-1β and caspase-11. The authors also provide compelling evidence linking the cytoplasmic LPS-sensing pathway to the recognition of Gram-negative bacteria, by monitoring caspase-11 activation in primed macrophages infected either with mutant *E. coli* that lacked biologically active LPS or with wild-type *E. coli*. Both normal and TLR4-deficient macrophages responded to the wild-type bacteria, but the mutant *E. coli* failed to drive responses that depended on caspase-11.

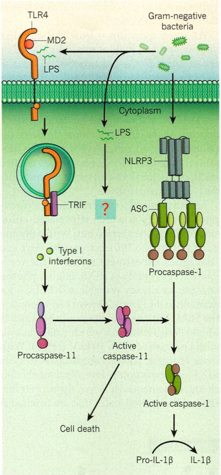

Figure 1 | Inflammasome activation by caspase-11. Infections with Gram-negative bacteria trigger the formation of an inflammasome protein complex comprising NLRP3, ASC and procaspase-1 in host cells. Active caspase-1 subsequently triggers the maturation of cytokines such as IL-1β. During responses to Gram-negative bacteria, including *Escherichia coli*, the progression of procaspase-1 to caspase-1 requires caspase-11. Caspase-11 expression is regulated by a signalling pathway involving the adapter protein TRIF and type-1 interferon proteins. This is initiated following the recognition of lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, by the cell-surface receptor TLR4 in complex with its co-receptor MD2. Kayagaki *et al.*[4] show that LPS can also be sensed in a TLR4-independent manner in the cytoplasm of host cells through an as-yet-unidentified receptor, and that this leads to the activation of caspase-11. In addition to its role in triggering caspase-1 activation, caspase-11 can cause host-cell death in a caspase-1-independent way.

It has been known for more than a decade that TLR4-deficient mice survive doses of LPS that are lethal to normal animals, and Kayagaki and colleagues' earlier work[5] found similar responses in mice that are missing caspase-11. The authors assessed the relevance of the cytosolic LPS-sensing pathway to these responses in TLR4-deficient or wild-type mice by first priming the mice with a non-lethal dose of a TLR3 ligand, to induce sufficient caspase-11 expression. Under these conditions, the TLR4-deficient mice were as susceptible as wild-type mice to LPS-induced septic shock.

Collectively, these findings provide compelling evidence for an LPS sensor in the cytoplasm that is involved in detecting Gram-negative bacterial infections, and highlight the importance of both TLR4 and this sensor in eliciting the severe inflammatory responses that result in LPS-induced mortality (Fig. 1). The study also reinforces an emerging theme in our understanding of antimicrobial defence: that multiple sensors recognize the same microbial product in a way that is specific to different cellular compartments. This strategy might help the host to gauge the severity of microbial invasion and tailor the response so that it is commensurate with the threat. For example, a tiny quantity of LPS will drive a TLR4-dependent proinflammatory response that alerts the host to the presence of infection. Higher quantities of LPS, which reach the cytoplasm, will trigger inflammasome activation, IL-1β production and, ultimately, cell death. This strategy might also be deliberate, because the cytoplasmic LPS-sensing pathway seems to be much more detrimental to the host than TLR4-based recognition. As a result, the cytoplasmic pathway would only be engaged following an overwhelming infection.

Several pressing questions arise from this study. The most crucial is: what is the identity of the sensor? We lack a detailed understanding of its ability to coordinate caspase-11 activation. Another key issue not yet addressed is whether cytoplasmic LPS triggers similar events in human cells. If it does, this understanding may be useful in the development of drugs for treating sepsis. For example, eritoran, a highly specific TLR4-inhibiting drug, recently failed in human clinical trials that aimed to reduce deaths from sepsis[14]; could this be because eritoran fails to block the cytoplasmic pathway of LPS recognition? About one-third of cases of septic shock result from Gram-negative bacterial infections, and these cases have a high mortality rate. A better understanding of the clinical relevance of the caspase-11 pathway in Gram-negative bacterial recognition in humans may improve the outcome of these infections. ■

Vijay A. K. Rathinam *and* **Katherine A. Fitzgerald** *are in the Division of Infectious Disease and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA.*
e-mail: kate.fitzgerald@umassmed.edu

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GEOCHEMISTRY

Sulphur from heaven and hell

Fingerprints of sulphur isotopes in rocks from the ridge beneath the Atlantic Ocean suggest that a substantial fraction of sulphur at Earth's surface is left over from the formation of the planet's core. SEE LETTER P.208

NICOLAS DAUPHAS

The relative abundances and isotopic signatures of sulphur found in rocks exhumed from Earth's mantle act as fingerprints of how and where our planet acquired its complement of this biochemically important element. Textbooks say that Earth's sulphur has the same isotopic composition as chondrites, a type of meteorite that is thought to best represent the building blocks of terrestrial planets. With dashes of audacity and insight, Labidi and co-workers[1] question this assumption in a paper on page 208 of this issue. They report measurements of rocks derived from Earth's mantle that reveal isotopic patterns unlike any others previously reported, with implications for how the planet formed*.

Most of us take Earth's chemical composition for granted, but unique circumstances may have been at play in delivering the right mix of elements for life to develop and flourish. For example, this is true of the noble metals needed in the electronic devices used to print or display this page. It is also true of sulphur, which is found in the amino acids cysteine and methionine and represents around 0.2% of our body weight. These elements and a few others

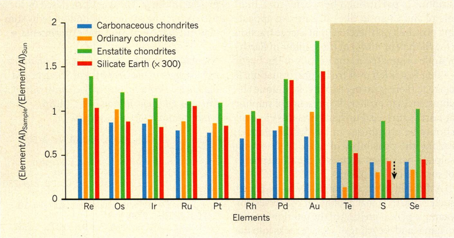

Figure 1 | Abundances of elements in Earth's mantle and in chondritic meteorites. The elements shown have strong affinities with metallic iron[9,11,12] and are in order of increasing volatility. Shaded region indicates elements that are not noble metals. Data are normalized to the abundance of aluminium (Al), because this element was neither lost to space during Earth's accretion nor scavenged by metallic iron during core formation. The data are also normalized to solar abundances to prevent them from spanning many orders of magnitude. The 'silicate Earth' pattern shows abundances in the mantle, and has been multiplied by a factor of 300 to allow easy comparison with the chondrite data. The depicted elements are thought to have segregated into Earth's core as the planet formed, but were subsequently replenished in the mantle by the accretion of chondritic material — which is why these elements are found in proportions similar to those of chondrites. However, Labidi *et al.*[1] suggest that about half or more of the sulphur in Earth's mantle may be left over from core formation (black arrow), with the remainder coming from chondrites; the upper part (light red) for sulphur represents the total abundance of the element in the mantle, and the lower part indicates the abundance left over from core formation.

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*This article and the paper under discussion[1] were published online on 4 September 2013.

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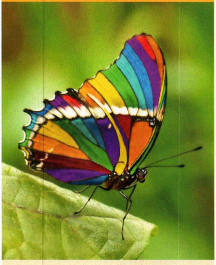

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have strong affinities for metallic iron — so much so that when Earth's metallic, iron-rich core formed, it scavenged all these elements, leaving behind a rocky mantle barren of sulphur, selenium, tellurium and noble metals. The prevailing view regarding the origin of these elements is that they were replenished in the mantle by a rain of asteroids from the heavens, known as the late veneer[2–5]. This late accretion of meteoritic material may also have delivered a fraction of the elements needed for life (hydrogen, carbon and nitrogen), as well as prebiotic organic molecules that could have served as the seeds for life.

The late-veneer hypothesis for the origin of Earth's sulphur is supported by strong observational evidence. First, laboratory experiments to reproduce the conditions of core–mantle segregation indicate that sulphur, selenium, tellurium and the noble metals are efficiently scavenged into metallic iron. Second, these elements are present in the mantle in proportions similar to those found in chondrites (Fig. 1). And third, the isotopic composition of sulphur in mantle rocks is identical to that of chondrites[6,7]. It is this third point that Labidi *et al.* call into question. They find that the ratio of sulphur-34 to sulphur-32 in Earth's mantle is 0.13% lower than that of chondrites.

To arrive at this conclusion, the authors used an analytical technique that provides a more complete recovery of sulphur from rock samples than has previously been possible. The difference in sulphur isotopic ratios that they infer between Earth's mantle and meteorites corresponds approximately to the difference measured in laboratory experiments when sulphur is partitioned between (core-like) metal and (mantle-like) silicate. An appealing possibility is therefore that a large fraction (maybe half or more) of the sulphur in Earth's mantle originated in the bowels of the Earth — that is, it is left over from core formation. If correct, there is no need to invoke unique circumstances to explain the presence of sulphur at Earth's surface. Furthermore, this element should be ubiquitous in Earth-like planets, raising the possibility of one day detecting sulphur-bearing molecules in the atmospheres of such extrasolar planets.

A difficulty with a dynamically active planet such as Earth is that geological processes, for example partial melting of the interior to form magmas and recycling of surface rocks into the interior at subduction zones, can blur isotopic signals and complicate interpretations. For instance, Labidi and co-workers identify the isotopic signatures of two distinct sulphur-containing components in rocks formed by melting of the mantle. One has a sulphur-isotope composition distinct from that of chondrites, which they interpret to be representative of the mantle. The other has a sulphur-isotope composition similar to that of chondrites, but the authors ascribe it to recycling of sulphur from sediments. This interpretation is reasonable, but a question lingers as to whether or not these components are representative of their mantle sources. During magma formation and extraction from the mantle, considerable amounts of sulphide minerals can remain behind at the magma's source, potentially affecting the sulphur isotopic ratios of magma-derived rocks[8]. Labidi *et al.* argue against this interpretation of their results, but insufficient experimental data are available on sulphur-isotope partitioning between sulphide and silicate melts to definitely rule out this possibility.

The relative abundances of selenium, sulphur and tellurium in the mantle have been used to gain insight into the nature of the late veneer[9]. These relative abundances best match the composition of carbonaceous chondrites (Fig. 1), suggesting that Earth received a late veneer of material rich in volatile compounds and organic molecules. Other isotopic evidence[10] suggests that the nature of the meteoritic material accreted by Earth was not very different before and after the completion of core formation. If Labidi and colleagues are correct and a substantial fraction of sulphur in the mantle is left over from core formation, this undermines the argument for a volatile-rich later veneer.

However, one is left wondering whether the good match between the selenium/sulphur and tellurium/sulphur ratios of chondrites and those of Earth can be coincidental. Labidi and co-workers' measurements are of the highest quality and will endure, but the same can be said of another study[9], published earlier this year, the conclusions of which contradict the present findings. The questions raised by these two conflicting studies will undoubtedly stimulate further discussion and experiments. ■

Nicolas Dauphas *is in the Origins Laboratory, Department of the Geophysical Sciences and the Enrico Fermi Institute, The University of Chicago, Chicago, Illinois 60637, USA.*
e-mail: dauphas@uchicago.edu

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NATURE

50 Years Ago

Prof. L. Egyed has recently summarized a number of hypotheses concerning the expansion of the Earth, and has suggested that the Earth's radius is expanding at a rate of 0.5–1.0 mm per year. There appears to be a remarkably close agreement between the rate of increase of the Earth's radius and that of the universe according to Hubble's law. Using the at present accepted value for Hubble's constant, $H = 100$ km/s/megaparsec, which is 1.65×10^{-4} mm per year per mile, and substituting the value of the Earth's radius in the Hubble equation, $v = RH$, we obtain a radial expansion for the Earth of 0.66 mm per year. While this agreement may be fortuitous it may suggest a fundamental concordance between expansion processes in the Earth's core and those responsible for the expansion of the universe.
From *Nature* 14 September 1963

100 Years Ago

In a paper on the psychology of insects, read before the General Malarial Committee at Madras in November, 1912, Prof. Howlett, after giving an account of experiments carried out by him on the response of insects to stimuli, comes to the conclusion that insects are to be regarded "not as intelligent beings consciously shaping a path through life, but as being in a sort of active hypnotic trance." It is claimed that this view of insect-psychology opens up great possibilities in the study of insect carriers of disease, since "it is no intelligent foe we have to fight, but a mere battalion of somnambulists." If we discover the stimuli or particular conditions which determine the actions of an insect, we can apply them to its undoing.
From *Nature* 11 September 1913

ASTROCHEMISTRY

Extracts of meteorite

LISA WARREN

The Sutter's Mill meteorite exploded in a dazzling fireball over California last year (pictured). Writing in *Proceedings of the National Academy of Sciences*, Pizzarello *et al.* report that it contained organic molecules not found in any previously analysed meteorites (S. Pizzarello *et al. Proc. Natl Acad. Sci. USA* http://dx.doi.org/10.1073/pnas.1309113110; 2013).

The authors' analysis of extracts of organic matter from the meteorite revealed a complex mixture of oxygen-rich compounds, which probably resulted from oxidative processes that occurred in the parent body. Intriguingly, the authors suggest that such compounds could have been released to prebiotic Earth during or after meteorite bombardments, adding to the roster of organic molecules that might have contributed to the evolution of life. **Andrew Mitchinson**

COMPUTATIONAL BIOLOGY

A recipe for ligand-binding proteins

Cellular cross-talk, enzymatic catalysis and regulation of gene expression all depend on molecular recognition. A method that allows the design of proteins with desired recognition sites could thus be revolutionary. SEE LETTER P.212

GIOVANNA GHIRLANDA

To support cellular processes, natural proteins have evolved to recognize a relatively small set of ligand molecules with high affinity and specificity. Broadening this set of protein–ligand pairs with synthetic proteins that are specific for ligands of choice could transform the development of biosensors, protein-based drugs, artificial enzymes and tools for chemical biology. Current *in vitro* approaches to the design of protein–ligand pairs rely on laborious directed-evolution techniques to engineer binding sites in existing protein scaffolds, and cannot be generalized. In this issue, Tinberg *et al.*[1] (page 212) describe a computational method that promises to streamline and greatly facilitate the development of tailored ligand-binding proteins*.

One way to engineer synthetic ligand-binding proteins is repurposing existing proteins through directed evolution. This approach involves selecting a few amino acids within an existing protein, typically located in a cavity, and randomly mutating them to create libraries of millions of protein variants, which are then selected for their ability to bind the target. Depending on the ligand and the scaffold protein, successive rounds of directed evolution yield binding proteins with dissociation constants in the physiologically relevant micromolar to nanomolar ranges[2,3]. The process is arduous, and success is not guaranteed: current methods limit the size of combinatorial libraries to about 10^9, which is often insufficient to explore a large number of positions and so limits the fitness of the resulting binders. Furthermore, the process works best when the starting protein already binds weakly to the desired ligand, rather than when starting from scratch.

Computational methods hold great promise to bypass this lengthy process by using virtual selection on a large number of scaffolds. Such approaches have enjoyed much success lately, with the design of artificial enzymes for a variety of reactions[4–8]. Despite these feats, the design of high-affinity ligand-binding proteins has remained elusive[9].

Tinberg *et al.* now present a generalizable strategy for designing artificial recognition modules for any desired target molecule. As their target they chose digoxigenin — a natural steroid that forms part of the cardiac drug digoxin, and that has found a second life as a recognition tag in biotechnology applications. Consequently, a few digoxigenin-binding agents, including both antibodies and non-antibody proteins, have been developed through traditional protein-engineering methods[10–12].

Taking some clues from the previously developed binding agents, Tinberg and colleagues sketched out a couple of possible ways to bind ligands to digoxigenin using amino acids that could provide hydrogen bonds and hydrophobic interactions (Fig. 1a). They then transposed these minimalistic sets into three-dimensional models, which were used as probes to interrogate a selected set of 401 proteins, encompassing several classes of

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*This article and the paper under discussion[1] were published online on 4 September 2013.

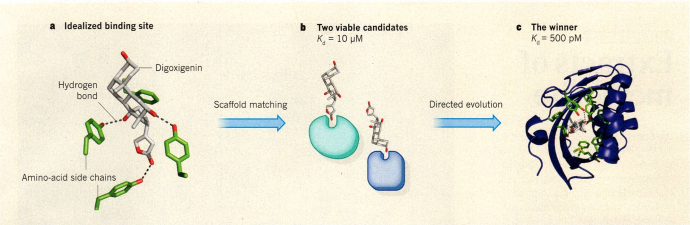

Figure 1 | Steps to making a ligand-binding protein. **a**, To design a protein with high binding affinity for the steroid digoxigenin, Tinberg *et al.*[1] created a minimal digoxigenin-binding site made with amino-acid side chains positioned to maximize hydrogen bonding and hydrophobic interactions to the target. They then sifted through a selected set of 401 scaffold-protein structures to find all (17) possible matches for the digoxigenin-binding site and ranked them by best fit. **b**, After validating the predictions through protein expression, they subjected the two best performers, which had micromolar binding affinities (K_d), to directed-evolution methods, randomizing amino acids farther away from the binding site. **c**, Finally, a protein with picomolar binding affinity emerged as winner, and the authors obtained its crystal structure to confirm the design features.

protein fold, for geometrical compatibility. Powerful computational methods available in the authors' lab enabled them to rapidly sift through all possible matches, rank the candidates and perform optimization through rounds of redesign and scoring.

Seventeen proteins emerged as candidates for experimental characterization, on the basis of optimal shape complementarity to the ligand, pre-organization of the site in the absence of ligand and predicted protein stability. Of these, only two bound the ligand with micromolar affinity (Fig. 1b). Although an impressive result for a computational approach, these binding affinities are far from the nanomolar ranges needed for practical applications. The authors, therefore, turned to directed-evolution methods to improve binding affinity.

To explore the effect of all 20 possible amino acids at each of 34 residues located within roughly 10 ångströms of the binding site would have required an astronomical number (more than 10^{44}) of possible protein sequences. Fortunately, a new method[13] allowed Tinberg *et al.* to circumvent this limitation — by randomizing each of the 34 positions independently, selecting for binding to labelled digoxigenin, and using deep sequencing to build a fitness map that correlates amino-acid identity at each position to function. On the basis of this information, the authors combined favourable substitutions in successive generations of libraries to obtain mutants with affinity in the low-nanomolar to high-picomolar ranges (Fig. 1c).

The litmus test for measuring successful protein design is obtaining high-resolution structures of the final product and comparing it with the designed features in the models. Tinberg and colleagues' proteins pass this test with flying colours: structural characterization of the two best mutants, DIG10.2 and its improved version, DIG10.3, showed the presence of hydrogen bonding between three designed tyrosine residues and the ligand. Tyrosine is frequently found in the binding pockets of antibodies and of synthetic ligand-binding proteins: its ability to form hydrophobic and hydrogen-bonding interactions, as well as its size and conformational rigidity, fulfil many roles in molecular recognition[14]. In addition to the designed features, subjecting the proteins to directed evolution allowed the exchange of small hydrophobic amino acids with larger ones, and the introduction of two additional tyrosines within the binding site. These mutations reinforce design principles incorporated in the computational selection, such as shape complementarity and binding-site pre-organization, which maximize the entropic contribution to binding as well as maximizing specificity.

Tinberg *et al.* also exploited hydrogen-bonding interactions, which impart strong geometric constraints and thus contribute to specificity for binding to digoxigenin as opposed to other steroids. This point is elegantly illustrated by a series of mutants in which the authors correlate the number of hydrogen-bonding groups on the target steroids with the effect of swapping each tyrosine for phenylalanine, which cannot hydrogen bond, and demonstrate the large energetic penalty paid for each mismatch.

The success of this protocol overcomes a long-standing issue in protein design, and promises to have an impact on the effectiveness of designed enzymes, which has often been limited by low affinity for the substrate. This work also opens up many exciting challenges. Digoxigenin proved a wise choice of target ligand, and contributed to the authors' success: the molecule offers several hydrogen-bonding 'hooks', which helped to achieve high binding specificity in the protein, and the steroid's rigid scaffold helped to maximize entropic contributions to binding. The authors were also able to gather information on what the binding site should look like from existing structures. Future work will, no doubt, implement ways to deal with flexible ligands and to design minimalistic binding sites in the absence of structural information. More powerful computational methods would enable affinity maturation *in silico* rather than *in vitro*, and would overcome the need for an existing scaffold as a starting point, by designing novel proteins around each target ligand[15]. Finally, molecular recognition could be coupled to functional activity, for example by using small-molecule binding to modulate protein activity. ■

Giovanna Ghirlanda *is in the Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287-1604, USA.*
e-mail: gghirlanda@asu.edu

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ARTICLE

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Social reward requires coordinated activity of nucleus accumbens oxytocin and serotonin

Gül Dölen[1]†, Ayeh Darvishzadeh[1], Kee Wui Huang[1] & Robert C. Malenka[1]

Social behaviours in species as diverse as honey bees and humans promote group survival but often come at some cost to the individual. Although reinforcement of adaptive social interactions is ostensibly required for the evolutionary persistence of these behaviours, the neural mechanisms by which social reward is encoded by the brain are largely unknown. Here we demonstrate that in mice oxytocin acts as a social reinforcement signal within the nucleus accumbens core, where it elicits a presynaptically expressed long-term depression of excitatory synaptic transmission in medium spiny neurons. Although the nucleus accumbens receives oxytocin-receptor-containing inputs from several brain regions, genetic deletion of these receptors specifically from dorsal raphe nucleus, which provides serotonergic (5-hydroxytryptamine; 5-HT) innervation to the nucleus accumbens, abolishes the reinforcing properties of social interaction. Furthermore, oxytocin-induced synaptic plasticity requires activation of nucleus accumbens 5-HT1B receptors, the blockade of which prevents social reward. These results demonstrate that the rewarding properties of social interaction in mice require the coordinated activity of oxytocin and 5-HT in the nucleus accumbens, a mechanistic insight with implications for understanding the pathogenesis of social dysfunction in neuropsychiatric disorders such as autism.

The mesocorticolimbic (MCL) circuit, implicated in encoding the rewarding properties of addictive drugs, is likely to have evolved to motivate behaviours that were important for survival and reproduction. Such incentive behaviours include eating, drinking and copulation, and are reinforced by so-called 'natural rewards' (for example, food, water, pheromones)[1]. Growing evidence suggests that social interaction itself can act as a natural reward[2]. However, given the diversity of social behaviours (for example, parental investment, mating, cooperation) and the selection pressures that shaped their emergence (reproductive, predation, limited resources)[3], it remains unclear whether evolutionarily conserved neural mechanisms exist to encode social reward.

An important clue comes from studies that have related pair-bonding behaviour in prairie voles (*Microtus ochrogaster*) to elevated expression of oxytocin receptors (OTRs) in the nucleus accumbens (NAc), a key component of the brain's MCL reward circuit[4]. However, the species-specific nature of this mating behaviour and the reported paucity of OTR expression in the NAc of mice[2,5,6] questions the relevance of NAc OTRs to consociate social behaviours. This topic is of particular interest given that polymorphisms in the OTR gene have been associated with autism spectrum disorders, which are characterized by profound social deficits, and may be amenable to treatment with oxytocin (OT)[7].

Mice are social animals: they live in consociate 'demes' consisting of five to ten adult members that share territorial defence[8] and alloparental responsibilities[9], and exhibit several behaviours (for example, vocal communication, imitation, and empathy)[10–12] that are the hallmarks of sociality. As in several other species including humans, OT has been linked to social behaviours in mice[7]. However, OT and OTR knockout mice show a number of related behavioural deficits (such as memory impairment, anxiety, stress, aggressivity)[5] that make it difficult to parse the function of OT as a social reward signal in the central nervous system. To examine the hypothesis that OT signalling in mice is required for the rewarding properties of social interactions, we used a conditioned place preference (CPP) assay that has traditionally been used to study the rewarding properties of drugs of abuse[13] and recently has been expanded to include social reward[14].

Social reward requires oxytocin

Male wild-type mice were conditioned for social CPP (Fig. 1a, b) while receiving intraperitoneal injections of either saline or the OTR antagonist (OTR-A), L-368,899 hydrochloride (5 mg kg^{-1}, twice a day for 2 days). Saline-treated wild-type mice showed a robust place preference for the socially conditioned context, whereas OTR-A treated mice showed no preference (Fig. 1c–e). Neither locomotor activity (Supplementary Fig. 1a–i) nor cocaine CPP (Supplementary Fig. 2a–d) was altered by OTR-A treatment, demonstrating the specificity of the effects of OTR-A for the social domain. Furthermore, OTR-A, but not saline, localized to the NAc using Andalman probes (Fig. 1f and Supplementary Fig. 3a, b) prevented social CPP (Fig. 1g–i), demonstrating that OT action in the NAc is required for consociate social reward.

Given the known species- and sex-specific variation in OTR expression[2,5,15,16], it is notable that no study so far[6,17–19] has determined whether hypothalamic OTergic inputs to the NAc exist in male mice. Here we injected recombinant rabies virus expressing enhanced green fluorescent protein (eGFP) (RBV-eGFP) into the NAc, where it is taken up by presynaptic terminals and retrogradely transported to cell bodies (Supplementary Fig. 4). In a substantial subset of hypothalamic neurons in the paraventricular nucleus (PVN), but not the supraoptic nucleus (SON), robust eGFP expression co-localizes with OT, indicating a direct axonal OTergic projection to the NAc (Supplementary Figs 4 and 5). Furthermore, these results suggest that it is the magnocellular projection from the SON that distinguishes prairie voles[6] from rats[19] and mice. Although they do not rule out an additional contribution of paracrine release, our findings demonstrate a significant synaptic source for OT in the NAc of male mice.

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[1]Nancy Pritzker Laboratory, Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, 265 Campus Drive, Stanford, California 94305, USA. †Present address: Department of Neuroscience, Johns Hopkins University, 855 North Wolfe Street, Baltimore, Maryland 21205, USA.

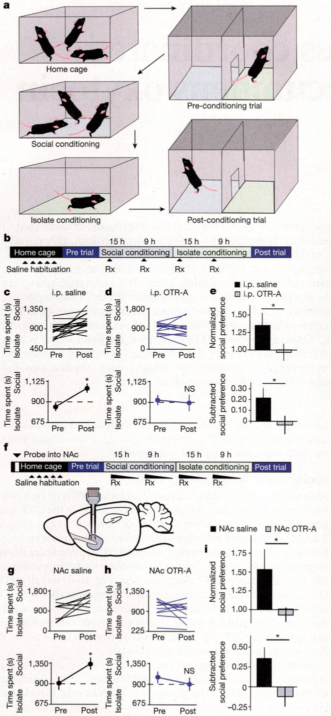

Figure 1 | Oxytocin is required for social CPP. a–i, Protocol for social CPP (**a**). Experimental time course of intraperitoneal (i.p.) injections (**b**) and NAc reverse microdialysis (**f**) in social CPP. Individual (top) and average (bottom) responses in animals receiving intraperitoneal (i.p.) (**c**) or NAc (**g**) saline versus animals receiving i.p. (**d**) or NAc (**h**) OTR-A. For both i.p. and NAc delivery routes, saline- but not OTR-A-treated animals spend more time in social bedding cue following conditioning ($n = 18$ i.p. saline, $n = 15$ i.p. OTR-A; $n = 9$ NAc saline and $n = 11$ NAc OTR-A animals). Values below 900 indicate that subjects preferred isolate bedding; values above 900 indicate that subjects preferred social bedding. **e, i,** Comparisons between treatment (Rx) groups reveal significantly decreased normalized and subtracted social preference in both i.p. and NAc OTR-A-treated animals. Summary data are presented as mean ± s.e.m. (*$P < 0.05$, Student's t-test). Each arrowhead indicates an application of saline or OTR-A.

Oxytocin induces presynaptic LTD in NAc MSNs

To interrogate directly the synaptic role of OT within the NAc, we recorded excitatory postsynaptic currents (EPSCs) from NAc medium spiny neurons (MSNs) in acute slices. Bath application of OT (1 μM, 10 min) caused a long-term depression (LTD) of EPSCs that was blocked (Fig. 2a–c) but not reversed (Fig. d–f) by the OTR-A L-368,899 hydrochloride (1 μM, continuous or 10 min application respectively). The magnitude of this oxytocin-induced LTD was significantly decreased in slices from socially conditioned versus isolation conditioned animals (Fig. 2g–i), consistent with the hypothesis that social experience elicits or influences the generation of OT-LTD.

To determine whether social experience preferentially influenced OT-LTD in one of the two major components of the basal ganglia circuit, direct (D1-receptor-expressing) versus indirect (D2-receptor-expressing) pathway MSNs[20], targeted recordings were made from NAc slices prepared from bacterial artificial chromosome (BAC) transgenic D1–TdTomato, and D2-eGFP reporter mice[21,22]. Application of OT induced robust LTD (Fig. 2j–l) and isolation conditioning resulted in increased magnitude of OT-LTD (Supplementary Fig. 6) in both D1- and D2-receptor-expressing MSNs, suggesting that these phenomena do not display direct and indirect pathway specificity.

To determine whether the OT-LTD was expressed pre- or postsynaptically, we performed a number of standard electrophysiological synaptic assays. The frequency, but not the amplitude, of miniature EPSCs, was significantly decreased by OT application (Fig. 2m–q). Furthermore, both the paired-pulse ratio (PPR) of EPSCs (50-ms inter-stimulus interval) and the coefficient of variation of the EPSCs increased following OT application (Fig. 2r–t). Together, these findings suggest that OT-LTD results from a decrease in presynaptic neurotransmitter release probability.

Social reward requires presynaptic OTRs in NAc

Anatomical studies have revealed sparse expression of OTRs in mouse NAc[2,5]. Moreover, immunostaining in OTR-Venus reporter mice[23] indicates that the small subset of cells that do express OTRs in the NAc are either inhibitory interneurons or glial cells (Supplementary Fig. 7). To test the hypothesis that OTRs in the NAc are preferentially localized to presynaptic boutons deriving from afferent inputs, we injected TdTomato-expressing RBV (RBV-TdTomato) into the NAc of OTR-Venus reporter mice (Supplementary Fig. 8). Cellular co-localization of TdTomato and Venus was detected in several, but not all, brain regions projecting to the NAc (Supplementary Fig. 8), identifying a number of putative sources of presynaptic OTRs in the NAc.

To extend the anatomical mapping of OTRs to their functional role in social reward *in vivo*, we used conditional OTR knockout mice[24] combined with Cre recombinase (Cre)-expressing RBV or adeno-associated virus (AAV) injected into the NAc, an approach that enabled selective ablation of pre- or postsynaptic NAc OTRs, respectively. Normal social CPP was observed in both sham-injected wild-type and conditional OTR mice (Fig. 3a–d). Injection of the AAV-Cre-eGFP to delete OTRs from cells within the NAc did not affect social CPP in either wild-type or conditional OTR mice (Fig. 3e–h). Consistent with this lack of effect of deleting OTRs from cells within the NAc, OT application did not induce long-lasting changes at inhibitory synapses onto MSNs (Supplementary Fig. 9). In contrast, injection of RBV-Cre-eGFP to delete presynaptic OTRs in the NAc, completely blocked social CPP in conditional OTR knockout mice but had no effect in wild-type mice (Fig. 3i–l). Injection sites and viral expression were confirmed for all animals (Supplementary Figs 10 and 11). Considered together with the pharmacological results showing OTRs within the NAc are required for social CPP (Fig. 1f–i), these results indicate that OTRs on presynaptic boutons within the NAc are required for social reward.

Social CPP and LTD require dorsal raphe inputs and 5HT1B receptors

To determine which of the afferent inputs expressing OTRs identified by RBV-mediated molecular ablation are required for social CPP,

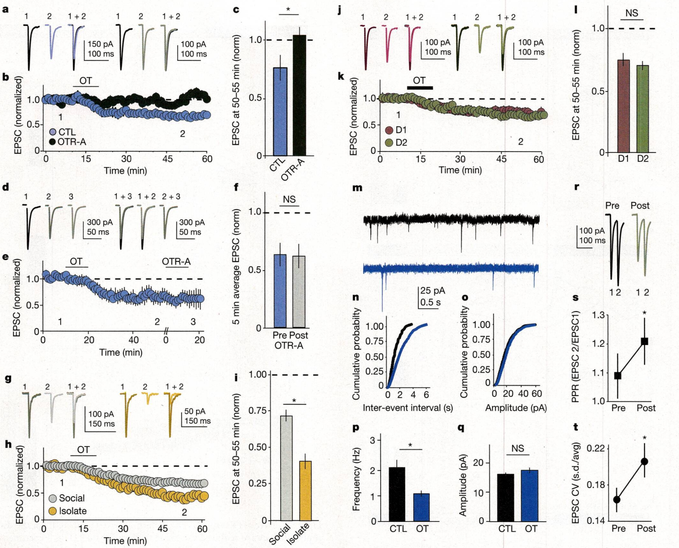

Figure 2 | Oxytocin induces LTD in the NAc. **a–h**, Representative traces (**a**, **d**, **g**, **j**), summary time course (**b**, **e**, **h**, **k**), and average post-treatment magnitude comparisons (**c**, **f**, **i**, **l**) reveal significant EPSC response depression in oxytocin-treated but not OTR-A-pre-incubated cells (**a–c**, $n = 6$ OT (oxytocin), $n = 6$ OT + OTR-A pre-incubation cells). OT-response depression is not reversed by post-induction OTR-A chase (**d–f**, $n = 7$ cells). The magnitude of OT-LTD is significantly increased in cells from isolation versus socially reared animals (**g–i**, isolate, $n = 14$, social $n = 27$ cells). The magnitude of EPSC OT-LTD is not different in D1 versus D2 MSNs (**j-l**, $n = 9$ D1, and $n = 11$ D2 cells). **m–q** Representative miniature EPSC traces (**m**), cumulative probability (**n**, **o**), and average (**p**, **q**) comparisons reveal that miniature EPSC frequency (**n**, **p**), but not amplitude (**o**, **q**), is decreased in OT-treated versus control cells (control, $n = 11$, OT, $n = 11$ cells). **r–t**, Comparisons of representative traces (**r**) and average (**s**) paired-pulse ratios PPR ($n = 6$ cells) as well as average (**t**) coefficient of variance, CV ($n = 32$ cells) reveal significant increases following induction of OT-LTD. Summary data are presented as mean ± s.e.m. (*$P < 0.05$, Student's t-test). Numbered traces (1, 2 and 3) were taken at the times indicated by numbers below the graphs.

we next injected AAV-eCre-eGFP into selected brain regions of conditional OTR mice. Deleting OTRs in either the anterior cingulate cortex or the ventral subiculum had no effect on social CPP (Supplementary Fig. 12), whereas AAV-Cre-eGFP injections into the dorsal raphe nucleus of conditional OTR mice, but not wild-type mice, prevented social CPP (Fig. 4a–d). This same manipulation also significantly reduced OT-LTD recorded *ex vivo* (Fig. 4e–g). Together these results provide support for the hypothesis that presynaptic OTRs on dorsal raphe nucleus axon terminals within the NAc are specifically required for social reward.

Since the dorsal raphe nucleus is one of the major sources of serotonin (5-HT) in the brain, we further characterized NAc projection neurons in the dorsal raphe nucleus and found substantial overlap between OTR- and 5-HT-expressing cells (Supplementary Fig. 13), raising the possibility of coordinated activity of these transmitters in the NAc. Given that 5HT1B receptors have been implicated in social behaviours[25,26] and autism[27], and their activation elicits a presynaptic LTD in the striatum[28], we reasoned that OT may induce LTD in the NAc through activation of 5HT1B receptors. Consistent with previous results[28], application of the 5HT1B selective agonist CP-93129 induced robust LTD in NAc MSNs (Fig. 5a–c). Subsequent application of OT caused no further depression (Fig. 5a–c), suggesting that the 5HT1B receptor-induced LTD had occluded OT-LTD. To test whether OT-LTD required release of 5HT within the NAc, we applied the 5HT1B receptor antagonist NAS-181 (20 μM) to NAc slices, a manipulation that largely prevented the LTD normally induced by OT (Fig. 5d–f). In contrast, 5HT1B receptor-induced LTD was readily induced in slices in which OTRs had been pharmacologically blocked (Fig. 5g–i) or molecularly ablated from dorsal raphe nucleus projections (Supplementary Fig. 14). Application of NAS-181 also prevented the decrease in miniature EPSC frequency normally elicited by OT (Fig. 5j–n), but had no effect on its own (Supplementary Fig. 15).

Together these results support the hypothesis that activation of OTRs on the terminals of dorsal raphe nucleus axons within the NAc leads to a 5HT1B receptor-dependent form of LTD (Supplementary Fig. 16) and that this synaptic modulation is necessary for social reward, as measured by social CPP. A strong prediction of this hypothesis is that

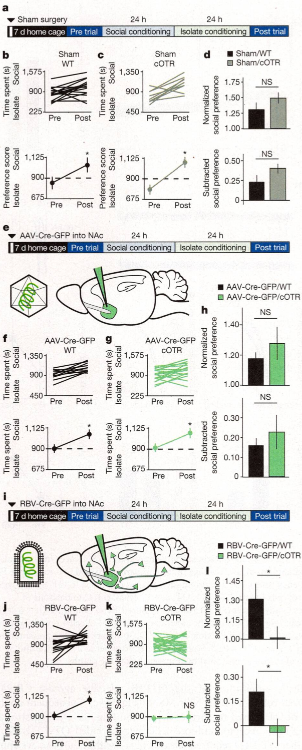

Figure 3 | Presynaptic OTRs are required for social CPP. a–l, Experimental time course for sham (**a**), NAc AAV-Cre-eGFP injection showing AAV particle and spread of Cre-eGFP from injection site (**e**), and NAc RBV-Cre-eGFP injection showing RBV particle and spread of Cre-eGFP from injection site (**i**). Individual (top) and average (bottom) responses in wild-type (WT) (**b, f, j**), versus conditional OTR (cOTR) (**c, g, k**) animals receiving sham (**b, c**), NAc AAV-Cre-eGFP (**f, g**) or NAc-RbV-Cre-eGFP (**j, k**). WT animals, as well as sham and NAc AAV-Cre-eGFP-injected cOTR animals, but not cOTR animals injected with NAc RBV-Cre-eGFP, spend more time in the social bedding cue following conditioning (sham WT, $n = 15$, cOTR, $n = 8$; NAc AAV-Cre-eGFP WT, $n = 15$, cOTR, $n = 19$; NAc RBV-Cre-eGFP WT, $n = 14$, cOTR, $n = 22$ animals). **d, h, l,** Comparisons between WT and cOTR animals reveal normal social CPP in sham and NAc AAV-Cre-eGFP-injected animals, whereas in NAc RBV-Cre-eGFP-injected animals social CPP is significantly decreased in cOTR versus WT controls. Summary data are presented as mean ± s.e.m. (*$P < 0.05$, Student's *t*-test).

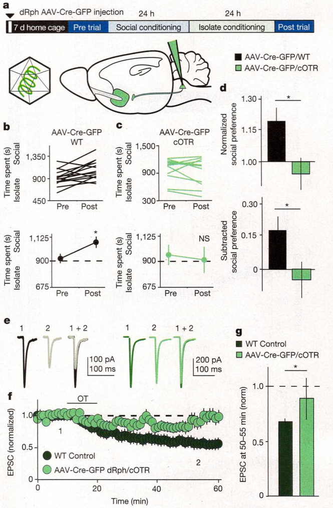

Figure 4 | NAc OTRs in presynaptic terminals originating from the dorsal raphe nucleus are required for social CPP and OT-LTD. a, Experimental time course of dorsal raphe nucleus (dRph) AAV-Cre-eGFP injections in social CPP. **b, c** Individual (top) and average (bottom) comparisons reveal that dRph AAV-Cre-eGFP-injected WT (**b**), but not cOTR (**c**) animals spend significantly more time in the social bedding cue following conditioning (WT, $n = 14$, cOTR, $n = 10$ animals). **d,** Comparisons between dRph AAV-Cre-eGFP-injected groups reveal significantly decreased social CPP in cOTR animals compared to WT controls. **e–g,** Representative traces (**e**), summary time course (**f**) and average post-treatment magnitude comparisons (**g**) reveal absence of OT-LTD in EPSCs recorded from dRph AAV-Cre-eGFP-injected cOTR knockout versus pooled WT control animals (dRph AAV-Cre-eGFP-injected cOTR, $n = 6$ cells; pooled WT control, $n = 30$ cells). Summary data are presented as mean ± s.e.m. (*$P < 0.05$, Student's *t*-test).

blockade of 5HT1B receptors within the NAc should prevent social CPP. Consistent with this prediction, NAS-181, but not saline, infusions into the NAc during conditioning (Fig. 6a) prevented the occurrence of social CPP (Fig. 6b–d).

Concluding remarks

We have demonstrated that the coordinated activity of OT and 5-HT is required for the reward associated with social interactions and modifies MCL circuit properties by generating LTD of excitatory synapses onto MSNs in the NAc. Moreover, our findings specifically implicate OT-mediated 5-HT release in the NAc in the regulation of social reward. Since OT-LTD occurs in both D1- and D2-receptor-expressing MSN subtypes, as does 5HT1B-LTD[28], these results suggest that social reward is not expressly governed by the dichotomies proposed by prevailing models of striatal function[20]. Indeed, the two-pathway framework for striatal function is almost certainly oversimplified[29–32] and computational

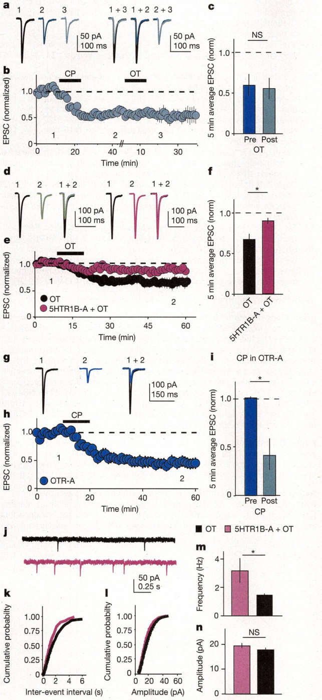

Figure 5 | OT-LTD in NAc requires 5HT1B receptors. a–i, Representative traces (**a**, **d**, **g**), summary time course (**b**, **e**, **h**) and average post-treatment magnitude comparisons (**c**, **f**, **i**) reveal that EPSC depression in cells treated with 5HTR1B agonist (CP-93129 dihydrochloride) is not augmented by subsequent application of OT (**a–c**, $n = 5$ cells); OT-LTD is significantly reduced in cells pre-treated with the 5HTR1b-antagonist (NAS-181) (**d–f**, control, $n = 7$, 5HTR1B antagonist, $n = 7$ cells); 5HTR1B-mediated LTD induced by application of CP-93129 is not affected by pharmacological blockade of OTRs (**g–i**, $n = 5$ cells). **j–n** Representative miniature EPSC traces (**j**), cumulative probability (**k**, **l**), and average (**m**, **n**) comparisons reveal miniature EPSC frequency (**k**, **m**), but not amplitude (**l**, **n**), is decreased in OT-treated cells versus cells treated with OT in the presence of NAS-181 (OT, $n = 17$, OT + 5HTR1b-A, $n = 17$ cells). Summary data are presented as mean ± s.e.m. (*$P < 0.05$, Student's *t*-test).

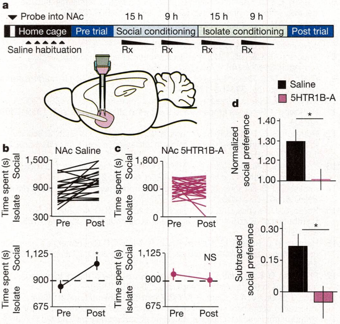

Figure 6 | Social CPP requires NAc 5HT1B receptors. a, Experimental time course of NAc reverse microdialysis. **b**, **c**, Individual (top) and average (bottom) responses in animals receiving NAc saline (**b**) versus 5HTR1B antagonist (5HTR1B-A) (**c**). Saline-treated animals, but not 5HTR1B-A-treated animals, spend more time in social bedding cue following conditioning (NAc saline, $n = 20$, NAc 5HTR1B-A, $n = 26$ animals). **d**, Comparisons between treatment groups reveal significantly decreased normalized and subtracted social preference in NAc 5HTR1B-A-treated animals compared to saline controls. Summary data are presented as mean ± s.e.m. (*$P < 0.05$, Student's *t*-test).

modelling studies have proposed that reinforcement learning engages multiple neuromodulatory reward circuits in parallel[33]. Furthermore, 5-HT and dopamine systems may represent reward in fundamentally different ways[34–36]. Future studies examining the interplay between dopamine and 5-HT in the regulation of social reward will therefore be informative.

In light of estimates that the shift to social living preceded the emergence of pair-living by 35 million years[3], we suggest that the NAc-dependent social reward mechanisms described here are the predecessors of evolutionary specializations seen in prairie voles[2,5,6]. These mechanisms utilize presynaptically localized OTRs, which couple to G-proteins[5], and thus may have been overlooked by previous studies that relied on receptor autoradiography and transcript tagging to conclude that OTRs do not exist in the NAc of consociate species like mice[2,6]. Moreover, as it is these antecedent social behaviours that are disrupted in neuropsychiatric diseases such as autism[37], the elucidation of the neural mechanisms mediating social reward is a critical step towards the development of rational, mechanism-based treatments for brain disorders that involve dysfunction in social behaviours.

METHODS SUMMARY

All procedures were conducted in accordance with the animal care standards set forth by the National Institutes of Health and were approved by Stanford University's Administrative Panel on Laboratory Animal Care. Male young adult mice (4 to 6 weeks of age) on a C57BL/6 background were used for all studies.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions G.D. and R.C.M. designed the study, interpreted results and wrote the paper. G.D. performed behavioural experiments, electrophysiology, and confocal microscopy. G.D., A.D. and K.W.H. performed stereotaxic injections and immunohistochemistry. K.W.H. generated RBV viruses. All authors edited the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.C.M. (malenka@stanford.edu).

ARTICLE

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RNAi screens in mice identify physiological regulators of oncogenic growth

Slobodan Beronja[1], Peter Janki[1], Evan Heller[1], Wen-Hui Lien[1], Brice E. Keyes[1], Naoki Oshimori[1] & Elaine Fuchs[1]

Tissue growth is the multifaceted outcome of a cell's intrinsic capabilities and its interactions with the surrounding environment. Decoding these complexities is essential for understanding human development and tumorigenesis. Here we tackle this problem by carrying out the first genome-wide RNA-interference-mediated screens in mice. Focusing on skin development and oncogenic (Hras^{G12V}-induced) hyperplasia, our screens uncover previously unknown as well as anticipated regulators of embryonic epidermal growth. Among the top oncogenic screen hits are Mllt6 and the Wnt effector β-catenin, which maintain Hras^{G12V}-dependent hyperproliferation. We also expose β-catenin as an unanticipated antagonist of normal epidermal growth, functioning through Wnt-independent intercellular adhesion. Finally, we validate functional significance in mouse and human cancers, thereby establishing the feasibility of *in vivo* mammalian genome-wide investigations to dissect tissue development and tumorigenesis. By documenting some oncogenic growth regulators, we pave the way for future investigations of other hits and raise promise for unearthing new targets for cancer therapies.

Genome-wide cellular RNA interference (RNAi) screening has advanced the identification of genes involved in oncogenic growth control. To date, however, high-throughput screens in mammalian cells have been limited to cultures, in which even the best systems incompletely model physiological environments. We have overcome this impediment by devising methods to efficiently and selectively transduce murine epidermis through *in utero* lentiviral targeting of progenitors in embryonic day (E)9.5 embryos[1]. When coupled with short hairpin RNA (shRNA) expression, lentiviral transduction is stably propagated throughout skin epithelium, resulting in RNAi-mediated reductions in target transcript and protein levels. This enables rapid analysis of complex genetic pathways in mammals, something previously only possible in lower eukaryotes[2–4].

The correlation between a tissue's growth and turnover rates and its susceptibility to cancer makes embryonic epidermis an attractive model for exploring how rapidly growing tissues balance proliferation and differentiation, and what prevents them from doing so in tumour progression. Given the efficacy of our system in single-gene studies, we have now expanded this scale by more than four orders of magnitude to conduct genome-wide RNAi screens. Our objectives were to first, demonstrate the feasibility of such screens in mammals; second, identify epidermal growth regulators in their native, physiological environment; third, uncover how epidermal growth control changes when it is propelled by a well-known oncogene; and fourth, demonstrate the implications of our findings for tumour progression in mice and humans.

Epidermal growth is rapid and uniform

Following completion of gastrulation and continuing to birth, mouse surface ectoderm commences rapid growth to match embryo expansion (Fig. 1a). Beginning as a monolayer, E9.5 ectoderm differentiates into a stratified, multi-layered epidermis that by birth constitutes a barrier that retains fluids and excludes microbes. Mature epidermis maintains an inner progenitor layer, which fuels tissue homeostasis and wound repair.

To quantify epidermal growth, we randomly marked single cells at clonal density by infecting E9.5 *Rosa26*$^{lox\text{-}stop\text{-}lox\text{-}(LSL)\text{-}yfp}$ Cre-reporter embryos (*R26*$^{yfp/+}$)[5] with an LV-Cre lentivirus[1], and then monitored their expansion during development (Fig. 1b). By E18.5, single yellow fluorescent protein (YFP)$^{+}$ cells at E10.5 had grown to clones constituting ~40 cells (Fig. 1b, c; ~5–6 divisions per cell). Variability in clone size ranged within 1–2 cell divisions, indicating strikingly uniform growth throughout the epidermis.

We next examined how growth is affected by oncogenic *Hras1*, which is found mutated in many cancers and is the primary target in skin carcinogenesis models[6,7]. *K14*-Cre[8]-driven, epidermal-specific expression of Hras^{G12V} from its endogenous locus (*Hras*$^{lox\text{-}wild\text{-}type\text{-}stop\text{-}lox\text{-}G12V}$)[9] resulted in mice whose skin displayed epidermal overgrowth as well as oncogenic Hras dose-dependency for one (*K14-cre; Hras*$^{LSL\text{-}G12V/+}$; Hras^{oncoX1}) or two (*K14-cre; Hras*$^{LSL\text{-}G12V/LSL\text{-}G12V}$; Hras^{oncoX2}) copies (Supplementary Fig. 1). Additional distinctions included the expansion of both progenitors (keratin 5^{+}) and differentiating layers (spinous keratin 10^{+}; granular filaggrin^{+}) (Supplementary Figs 1–3).

To quantify the impact of oncogenic Hras on epidermal growth, we used a cellular growth index (CGI) assay[1] (Fig. 1d). Cre-reporter (*R26*$^{yfp/+}$) embryos, transduced with a LV-Cre and red fluorescent protein-expressing lentivirus (LV-RFP) mix showed similar relative numbers of YFP^{+} to RFP^{+} keratinocytes across several embryos, indicating that control YFP^{+} and RFP^{+} populations grew at comparable rates (Fig. 1e, f). Transduction of the same lentiviral mixture into test animals (where Cre-transduction induces Hras^{G12V}) revealed consistently more YFP^{+} (Hras^{G12V}) than RFP^{+} (control) cells (CGI = 1.82, Hras^{oncoX1}; 3.32, Hras^{oncoX2}; Fig. 1e, f). These findings demonstrate that Hras^{G12V} confers a dose-dependent growth advantage to skin epidermis and that growth rates can be documented and quantified.

Hras^{G12V} not only conferred a dose-dependent increase in proliferation, but also in suppression of apoptosis (Supplementary Fig. 4). In addition, consistent with established pro-inflammatory effects of Hras^{G12V}, innate and adaptive immune cells infiltrated underlying dermis. Last, real-time PCR revealed no evidence for oncogenic induction of cellular senescence-associated cyclin-dependent kinase (CDK) inhibitors[10].

Establishing pooled screen parameters

On the basis of our CGI assay principle, we expected that, after epidermal transduction with a pool of shRNA-expressing lentivirus, any

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[1] Howard Hughes Medical Institute, Laboratory of Mammalian Cell Biology & Development, The Rockefeller University, New York, New York 10065, USA.

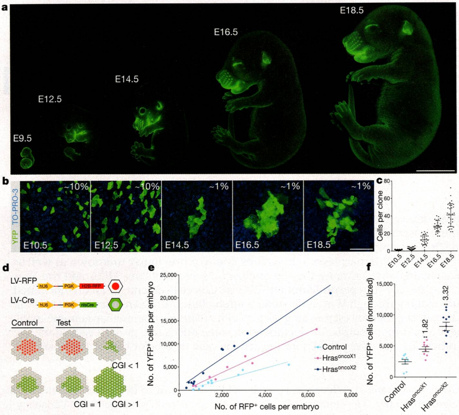

Figure 1 | Embryonic epidermal tissue growth is rapid and responsive to oncogenic Hras. **a**, Mouse embryogenesis, highlighted by propidium iodide (E9.5) or K14-actin–GFP (E12.5–18.5). **b**, *R26*$^{yfp/+}$ Cre-reporter embryos infected at E9.5 with LV-Cre and analysed at days shown. Transduced cells are YFP$^+$. Transduction levels (% YFP$^+$ cells) depend upon viral titre. **c**, Cell numbers in transduced YFP$^+$ clones at ages shown. **d**, Schematic of CGI assay. E9.5 *R26*$^{yfp/+}$ Cre-reporter (control) or *gene*$^{lox/lox}$ *R26*$^{yfp/+}$ (test) embryos are infected with a LV-Cre and LV-RFP mix. At E18.5, numbers of RFP$^+$:YFP$^+$ cells in control and test animals are compared, and phenotypes scored as neutral (CGI = 1), growth advantaged (CGI > 1) or disadvantaged (CGI < 1). **e**, Numbers of RFP$^+$ and YFP$^+$ cells at E18.5 in control, Hras$^{\mathrm{oncoX1}}$ and Hras$^{\mathrm{oncoX2}}$ embryos. Upper shift is consistent with growth advantage. **f**, RFP$^+$ cell numbers normalized to YFP$^+$ cells in control, Hras$^{\mathrm{oncoX1}}$ and Hras$^{\mathrm{oncoX2}}$ animals. CGI assay suggests a 1.8-fold overgrowth ($P = 0.002$) in Hras$^{\mathrm{oncoX1}}$ and 3.3-fold overgrowth ($P < 0.0001$) in Hras$^{\mathrm{oncoX2}}$ epidermis. Error bars indicate ± s.d (**c**) and ± s.e.m (**f**). **($P \leq 0.01$) in **f** indicates statistical significance of comparison to control. For CGI assay (**e**, **f**), data points are individual embryos: control ($n = 9$), Hras$^{\mathrm{oncoX1}}$ ($n = 8$) and Hras$^{\mathrm{oncoX2}}$ ($n = 11$). Scale bars, 5 mm (**a**) and 50 μm (**b**).

shRNA that targets an essential mediator of growth will be reduced or lost during development, whereas shRNAs targeting negative growth regulators will become overrepresented. By comparing relative shRNA abundance in the initial pool and at E18.5, we expected to identify shRNAs and their targets that confer either growth advantage or disadvantage.

The success of the approach depended upon our ability to: (1) modify growth at a low multiplicity of infection (MOI ≤ 1); (2) measure individual shRNA abundance in the pool; (3) transduce embryonic epidermis at a MOI ≤ 1; and (4) achieve complete screen coverage, in which every shRNA in the pool is tested. We set up a series of controls to ensure that these parameters were met. Underscoring the feasibility of pooled-formats for *in vivo* RNAi screens, we demonstrated that targeting of (1) anaphase promoting complex component *Anapc5* during normal growth, and (2) *Hras1* during oncogenic hyperplasia, reduced average clone sizes, even with transductions where most cells harboured only a single shRNA (Supplementary Fig. 5).

To quantify individual shRNA representation in a complex pool, we used the Illumina-based count-by-sequencing principle (Supplementary Fig. 6). We designed oligonucleotides to amplify the target sequence of each shRNA, and optimized pre-amplification and clean-up pipelines to yield a product to apply directly to the sequencing cell. We tested our protocol against a defined template generated by combining genomic DNAs from independently transduced cell lines, so that individual genome-integrated shRNAs were present in amounts corresponding from a single cell (6 pg) up to 2,048 cells (12.3 ng).

We amplified and sequenced this standard set, and showed that reactions were: (1) quantitative, with increased sequencing reads corresponding to shRNA abundance in the pool; (2) sensitive, detecting all three single-copy shRNAs; and (3) highly reproducible (Supplementary Fig. 6). Independent counts of the standard set showed identical sequencing bias for a given shRNA, and thus became neutralized in relative comparisons of absolute counts, especially with ≥32 copies of the shRNA. Indeed, a >30-fold screen coverage proved sufficient to sample all shRNAs in our pool (see below). At this level, growth-neutral shRNAs were >1,000-fold represented in the E18.5 sequencing quantification reaction, because each E9.5 epidermal cell generates ~40 cells by E18.5.

We next determined that at an infection level of 13–27%, most transduced epidermal keratinocytes carried a single lentivirus (MOI ≤ 1) (Supplementary Fig. 7). To ensure that at least 30 individual cells were infected with each shRNA at E9.5, a pool of ~78,000 shRNAs required ~10^6 cells to be targeted. We used high-resolution imaging of TO-PRO3-labelled embryos and established that at E9.5, surface ectoderm contained ≥120,000 cells per embryo. Together, this suggested that transducing ≥90 embryos would achieve the requisite coverage (Supplementary Fig. 8).

Screens identify known growth regulators

We pooled the genome-wide collection of murine shRNA lentiviruses in roughly equal concentrations[11], and profiled the starting composition of the pool ($t = 0$) in transduced primary mouse keratinocytes (Fig. 2a). Physiological screens were performed in control and Hras$^{\mathrm{oncoX2}}$ embryos transduced at E9.5 *in utero* (Fig. 2b). Epidermal cells were collected after 24 hours (initial pool) and 9 days of development, and integrated lentiviral hairpins from genomic DNAs were sequenced and quantified (Fig. 2c).

Our pre-amplification and sequencing reactions did not bias shRNA quantification, and Illumina sequencing reads were of high quality. They mapped to the shRNA library with predictable efficiency and indicated complete coverage of the pool (Supplementary Figs 9, 10). Significantly altered shRNAs were identified and ranked on the basis of two independent methods. To ensure reproducibility, we used DESeq statistical package[12], which accounted for biological variability among our replicates (sets of 30 embryos per condition) and is the best method to identify candidates per se (Fig. 2d–k). However, informative yet variable shRNAs can be excluded by the high stringency of DESeq, which reduces the ability to control for off-target effects by requiring that multiple shRNAs show consistent behaviour. We therefore also analysed pooled data sets (90 embryos per condition;

Supplementary Fig. 11) using Fisher's exact test, which reduces variability by averaging individual shRNA abundance. By maximizing screen coverage, this method produces a more inclusive list of significantly altered shRNAs, and hence was preferred for ranking candidates identified by DESeq (Fig. 2f, j, k). Importantly, the overlap between these approaches was extensive (Supplementary Fig. 11), underscoring the robustness of our data.

We identified ~1,800 genes as essential for normal growth (Fig. 2d, e and Supplementary Tables 1, 2) and significantly enriched for function in protein synthesis ($P = 3.1 \times 10^{-10}$) and gene expression ($P = 2.6 \times 10^{-10}$). Genes encoding 60S and 40S ribosomal proteins were also highly represented and among the top 10% of all hits for normal growth, underscoring our screen's power to identify regulators of normal growth/viability (Fig. 2f and Supplementary Fig. 12). Indeed, our top 10 candidates for regulators of normal growth featured six ribosomal genes and two genes essential for messenger RNA splicing (Fig. 2j).

In our screen for specific regulators of Hras^{G12V}-dependent oncogenic growth, ~160 genes surfaced as candidates (Fig. 2g and Supplementary Tables 3, 4). They diverged in identity and function from those implicated in normal growth regulation, as most housekeeping growth regulators were eliminated by pair-wise comparison of control and Hras^{oncoX2} shRNA abundance (Fig. 2h). The oncogene-specific regulators included Hras1, and downstream Ras pathway members Raf1, and Mek and Akt proteins (Fig. 2i). Equally notable was the absence of upstream oncogenic Ras regulators, for example, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which are not expected to arise in a screen for hairpins suppressing Hras^{G12V}-induced growth. Our top 10 hits for regulators of oncogenic growth included well-established Ras pathway members *Akt3* and *Raf1*, as well as the Ras-regulated *Pawr*[13] (Fig. 2k). Cyclin C (*Ccnc*), a putative regulator of haematopoietic stem cell quiescence[14], was also on this list, as was *Mllt6*, encoding a myeloid/lymphoid, or mixed-lineage, leukaemia (MLL) translocation partner and a component of an epigenetic modifier complex[15].

The very top candidate for preferential regulation of oncogenic growth was *Ctnnb1*, encoding the Wnt signalling and intercellular adhesion effector β-catenin. At first glance, β-catenin seemed obvious, as its over-activation has been implicated in a variety of cancers, including those of skin[16–18]. However, β-catenin is also thought to be essential for stem cells[19]. Hence, it was surprising that its hairpins surfaced in a screen for selective inhibitors of oncogenic and not normal growth. Even more paradoxical was that in our parallel genome-wide screen, *Ctnnb1* was the top candidate for negative regulation of normal epidermal growth (Supplementary Tables 1, 2).

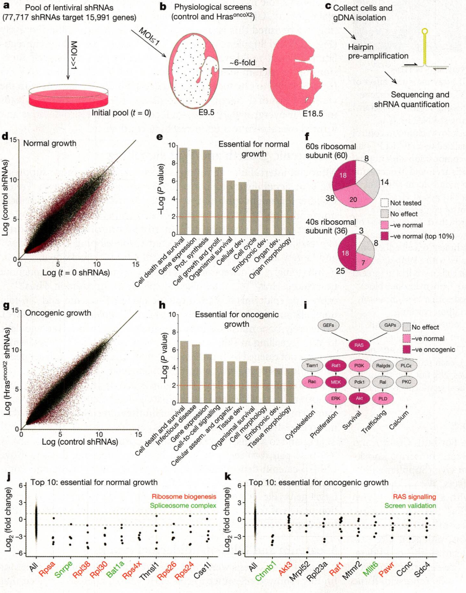

Figure 2 | Genome-wide RNAi screens for physiological regulators of normal and oncogenic growth identify expected and surprising regulators. a–c, Schematic of the RNAi screens based on relative enrichment/depletion of individual shRNAs over time. **a,** shRNAs against 15,991 mouse genes are combined into a lentiviral pool whose composition is determined from the 'initial pool' ($t = 0$) experiment, in which transduced cells are analysed 24 h after infection. **b,** Genes that regulate normal and oncogenic growth are identified in two screens, in which E9.5 control or K14-Cre$^+$; Hras^{oncoX2} embryos are infected *in utero*, allowed to develop for 9 days, and processed. **c,** After epidermal tissues are collected and used in genomic DNA (gDNA) isolation, individual shRNAs are pre-amplified and quantified by sequencing unique hairpin regions. **d–k,** Significantly enriched/depleted shRNAs identified using DESeq analysis. **d,** Dot plot of relative abundance of 77,717 shRNAs at $t = 0$ and in E18.5 epidermis. **e,** Putative normal growth regulators are significantly enriched ($P \geq 0.01$) for gene function categories promoting cell viability and development. **f,** Normal growth regulators encode for ribosomal 60S and 40S subunit components ($P = 1.12 \times 10^{-9}$ and $P = 3.26 \times 10^{-7}$, respectively), with many in the top 10% of all hits (maroon). **g,** Relative shRNA abundance in control and Hras^{G12V} animals reveal oncogenic growth-altering shRNAs. **h,** Putative oncogenic growth regulators are enriched ($P \geq 0.01$) in gene categories that support cell growth. **i,** Downstream effectors of Ras signalling score as essential for growth (pink), with many exhibiting an oncogene-specific requirement (maroon). **j, k,** shRNAs for the top ten essential regulators of normal (**d**) and oncogenic (**g**) growth are markedly depleted relative to the genome-wide pool (all).

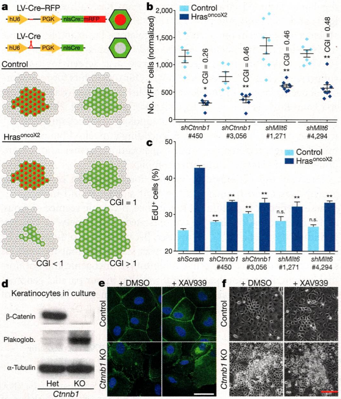

Figure 3 | Suppressing β-catenin and Mllt6 selectively affects Hras^{G12V}-dependent epidermal hyperplasia. **a**, Modified CGI assay measures effect of shRNA-mediated gene knockdown in animals with Cre-activated transgene expression. Transduction with LV-Cre–RFP co-expressing scrambled (scram) shRNA, and LV-Cre co-expressing candidate-targeting shRNA, leads to generation of YFP$^+$ RFP$^+$ scrambled and YFP$^+$ knockdown clones in control or Hras^{G12V} animals. Numbers of YFP$^+$ cells (normalized to YFP$^+$ RFP$^+$) in control and Hras^{oncoX2} animals reflect lentiviral mix composition after normal and oncogenic growth, respectively. **b**, Fewer YFP$^+$ cells are found in oncogenic animals upon knockdown of *Ctnnb1* and *Mllt6* with independent shRNAs. **c**, Reduced EdU incorporation following *Ctnnb1* knockdown in Hras^{G12V} animals contrasts with increased proliferation in control epidermis. *Mllt6* depletion also reduces EdU labelling in oncogenic growth. **d**, Immunoblot of control (*Ctnnb1* heterozygous (Het)) and *Ctnnb1* knockout (KO) keratinocyte lysates shows upregulation of plakoglobin. **e**, Establishment of cell adhesion 48 h after Ca^{2+} shift is unaffected in keratinocytes treated with a Wnt-signalling inhibitor (XAV939) but impaired in *Ctnnb1* knockout cells. E-cadherin (green) marks adherens junctions and DAPI (4′,6-diamidino-2-phenylindole; blue) labels the nuclei. **f**, Unlike control cells or cells treated with a Wnt-inhibitor, *Ctnnb1* knockout keratinocytes form overgrown foci upon reaching confluence. Error bars (**b**, **c**) indicate ± s.e.m. Data points (**b**, **c**) represent individual embryos with $n = 6$ (*shCtnnb1* and *shScram* in control), $n = 7$ (*shMllt6* no. 4,294 in control), $n = 8$ (*shMllt6* no. 1,271), $n = 9$ (*shMllt6* no. 4,294 in Hras^{G12V}), or $n = 10$ (*shScram* in Hras^{G12V}), each scored through immunofluorescence analysis of ten 425.1 μm^2 images. NS, not significant ($P > 0.05$); $^*P \leq 0.05$ and $^{**}P \leq 0.01$ indicate statistical significance. Scale bars, 50 μm (**e**), 10 μm (**f**).

Validating oncogene-specific regulators

We chose *Ctnnb1* and *Mllt6* for further study. For both *Ctnnb1* and *Mllt6*, a direct correlation was observed between transcript knockdown *in vitro* and severity of growth defects *in vivo* (Supplementary Fig. 13), strongly arguing against off-target effects. We validated our candidates as oncogenic growth regulators with a modified *in vivo* CGI assay involving two lentiviral vectors (Fig. 3a). In one, Cre-recombinase fused to monomeric RFP (LV-Cre–mRFP) contained a scrambled shRNA control. In the other vector, untagged Cre was used; this vector encoded the test shRNA against the candidate. Transduction of E9.5 control or Hras^{oncoX2} Cre-reporter embryos marked two separate populations: RFP$^+$ YFP$^+$ cells represented the baseline rate of normal (in control) or oncogenic growth (in Hras^{oncoX2}); YFP$^+$ cells represented the rate of growth that occurs when the target transcript is depleted.

The ratios of YFP$^+$ cells normalized to YFP$^+$ RFP$^+$ cells in Hras^{oncoX2} and control animals revealed that two independent *Ctnnb1* shRNAs displayed reductions of ~2–4-fold in oncogenic relative to normal growth. Similar ~2-fold reductions in YFP$^+$ cells were observed in the Hras^{oncoX2} background when *Mllt6* transcripts were diminished (Fig. 3b). The physiological effects of *Ctnnb1* or *Mllt6* knockdown were profound: the neonatal oncogenic phenotype was significantly ameliorated, and epidermal proliferation in Hras^{oncoX2} embryos was markedly and reproducibly suppressed. By contrast, no significant effects were seen on apoptosis (Fig. 3c and Supplementary Fig. 13).

Equally interesting to the selective effects of *Ctnnb1* knockdown on suppressing Hras^{G12V}-dependent oncogenic growth were its positive effects on normal growth. These differences seemed to be physiologically relevant, as they were reflected at the level of EdU-incorporation and thickness of epidermal tissue (Fig. 3c and Supplementary Fig. 14). Although hitherto overlooked, the proliferative effects of *Ctnnb1* hairpins on normal epidermis are recapitulated upon conditional targeting of β-catenin[20,21].

β-Catenin is both an adherens junction component and a nuclear cofactor for Wnt regulators in the LEF/TCF family and other DNA-binding proteins[22]. However in contrast to its nuclear functions, β-catenin's role in adhesion has been assumed to be redundant with plakoglobin[23]. Given that intercellular defects can promote proliferation, we revisited this issue using a sensitive *in vitro* adhesion assay (Fig. 3d–f and Supplementary Fig. 15)[24]. Despite plakoglobin upregulation, *Ctnnb1*-null keratinocytes inefficiently formed cell–cell adhesions upon calcium induction. Moreover, the Wnt inhibitor XAV939 (ref. 25) failed to phenocopy these defects. Finally, consistent with the view that loss of β-catenin compromises contact inhibition and leads to cellular overgrowth, *Ctnnb1*-null cells were hyperproliferative and formed overgrown foci upon reaching confluence.

Oncogenic growth and Wnt signalling

Although intercellular adhesion is often viewed as tumour suppressive, Wnt signalling is often associated with oncogenic growth. To test whether this might contribute to the negative effects of *Ctnnb1* knockdown on Hras^{G12V} skins, we transduced embryos with both a Wnt-reporter and LV-Cre (Fig. 4a). In E18.5 control animals, reporter expression was predictably restricted to developing hair follicles[26] and largely abolished with concomitant *Ctnnb1* knockdown.

Intriguingly, Wnt-reporter expression was expanded throughout transduced Hras^{oncoX2} interfollicular epidermis. In addition, Hras^{G12V}-expressing epidermis displayed ectopic nuclear β-catenin and >6-fold upregulation of *Axin2* transcripts (Fig. 4a and Supplementary Fig. 16a–c). Conversely, bone morphogenetic protein signalling, which is antagonistic to Wnt signalling in skin[27], was downregulated in Hras^{GV12} epidermis, and was not rescued by β-catenin depletion, suggesting its independence of Wnt in this oncogenic context (Supplementary Fig. 18).

Mllt6 expression paralleled β-catenin and Wnt-reporter activity, both in normal hair follicles and in evaginating structures of Hras^{oncoX2} skin (Supplementary Fig. 16b). Chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) analysis showed that Tcf3 and Tcf4 bound to a conserved Lef/Tcf motif upstream of *Mllt6* (Fig. 4b). A 299-base-pair (bp) segment (green line) encompassing this site drove LEF1/β-catenin-dependent luciferase reporter activity in a manner comparable to the 331-bp Tcf3/4 binding site of *Axin2*, an established Wnt-target gene (Fig. 4c and Supplementary Fig. 16d). In agreement, depletion or loss of β-catenin in embryonic epidermis *in vivo* reduced *Mllt6* transcript levels (Fig. 4d).

β-catenin and Mllt6 in epidermal tumours

Although our screens were conducted on embryonic mouse skin, our findings showed relevance to cancer. RNA-seq analysis revealed that

Ctnnb1 or *Mllt6* depletion in oncogenic Hras epidermis affected a shared set of transcripts ($P = 2.48 \times 10^{-40}$ and $P = 8.16 \times 10^{-25}$) that globally suppressed pathways promoting tumorigenesis (for example, *Myc*, *E2f1*) and enhanced those restricting growth (for example, *Trp53*, *Cdkn2a*; Fig. 4e, f). Moreover, in human squamous cell carcinomas (SCC), β-catenin and MLLT6 were often upregulated and nuclear (notably in the basal layer, where cancer stem cells reside[28]). Our analysis of 75 different human skin SCCs showed that most tumours expressed both proteins, with significant correlation in their expression (Supplementary Fig. 18). It remains to be seen whether their co-expression in tumours and a shared effect on transcriptional profile during oncogenic growth reflects a functional interaction between our candidates, or is a result of their independent effect/importance on the cellular machinery at the heart of oncogenic growth.

We next tested whether β-catenin and Mllt6 are physiologically relevant to Hras^{G12V}-dependent tumour initiation and maintenance. Whereas clonal LV-Cre-mediated activation of Hras^{G12V} expression in mice resulted in squamous papilloma formation as early as 3 weeks of age, concomitant constitutive expression of *Ctnnb1* or *Mllt6* shRNAs delayed tumour initiation (Fig. 5a). Moreover, growth of orthotopically transplanted SCC cells was significantly reduced following candidate depletion (Fig. 5b and Supplementary Fig. 19).

We extended this physiological relevance by performing xenografts of human SCC cells transduced with lentivirus harbouring scrambled or shRNAs targeting human *CTNNB1* or human *MLLT6*. Tumour initiation was significantly delayed, with *MLLT6* showing stronger effect than *CTNNB1* (Fig. 5c and Supplementary Fig. 19).

Finally, to assess whether *Ctnnb1* and *Mllt6* are required for tumour maintenance, we engineered an LV-Cre vector that allows for doxycycline-regulated shRNA expression, thereby enabling induced depletion of *Mllt6* and *Ctnnb1* following tumour formation in adult animals (Fig. 5d and Supplementary Fig. 20). Both had negative effects on tumour maintenance, with some tumours showing partial regression. Thus, the tumour-suppressive effects of *Ctnnb1* and *Mllt6* shRNAs as first revealed in embryogenesis appeared to be functionally relevant to adult tumorigenesis.

Discussion

The urgent need to understand cancer has fuelled human cancer genome sequencing and *in vitro* RNAi-based screening efforts to

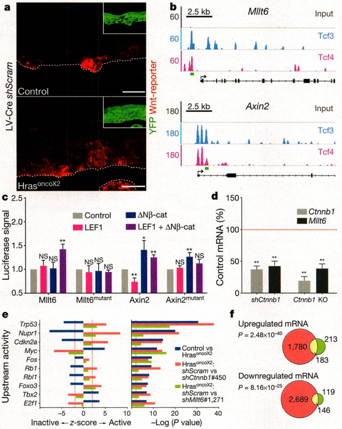

Figure 4 | Hras^{G12V}-induced epidermal growth affects other signalling pathways. a, Wnt-reporter activity (red) is restricted to hair placodes in control skin (top) but extends to interfollicular epidermis in Hras^{oncoX2} animals (bottom). YFP (inset) marks LV-Cre-transduced epidermis. White dotted line demarcates dermal–epidermal boundary. **b,** ChIP-seq peaks on chromosome 11 reveal Tcf3 (blue) and Tcf4 (pink) binding sites in *Mllt6* and *Axin2* promoter regions. Negative control (grey) is total genome DNA. Green bars represent the ~300-bp fragment used to validate Tcf3/4 binding. **c,** Human LEF1 (TCF3/4 family member) and stabilized β-catenin (ΔNβ-cat) together promote luciferase activity when putative TCF3/4 binding sites of *Mllt6* and *Axin2* are used as drivers. These effects are not observed when the TCF3/4 binding motifs are mutated. **d,** *Mllt6* and *Ctnnb1* mRNA epidermal levels are reduced by *Ctnnb1* knockdown (*shCtnnb1* no. 450) or knockout (KO). **e,** Transcriptional profile of Hras^{G12V} epidermal progenitors reveals repression of tumour suppressors (for example, *Trp53*, *Cdkn2a*, *Rb1*) and activation of oncogenic signalling (for example, *Myc*, *E2f1*). shRNA-mediated depletion of *Ctnnb1* or *Mllt6* in Hras^{G12V} epidermis significantly counter these transcriptional changes. Red vertical lines represent significant activation *z*-score (twofold) and *P* value of a correct prediction ($P = 0.01$). **f,** Significant overlap in differentially regulated transcripts is observed following depletion of β-catenin and Mllt6 in Hras^{G12V} epidermal progenitors. Error bars indicate ± s.e.m. (**c**) and ± s.d. (**d**). In the real-time PCR experiment (**d**), data are shown for three embryos assayed in two independent reactions ($n = 6$). $^*P \leq 0.05$, $^{**}P \leq 0.01$. Scale bars, 50 μm.

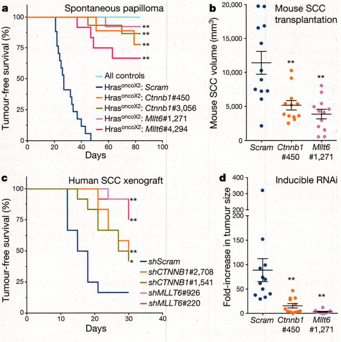

Figure 5 | β-catenin and Mllt6 depletion impair Hras^{G12V}-dependent tumorigenesis. a, shRNA-mediated depletion of *Ctnnb1* or *Mllt6* delays spontaneous tumour initiation in Hras^{oncoX2} mice ($n = 9$ in all conditions except LV-Scram-transduced Hras^{oncoX2} ($n = 18$)). Control lines correspond to animals transduced with shRNAs with no effect on tumorigenesis. **b,** Tumour volumes of *Ctnnb1*- and *Mllt6*-depleted mouse SCCs transplants are significantly reduced after 30 days of growth. **c,** Tumour initiation following xenotransplantation of shRNA-transduced human SCC cells is significantly delayed following knockdown of human *CTNNB1* or *MLLT6*. **d,** Induction of *Ctnnb1* or *Mllt6* knockdown in pre-existing spontaneous mouse papillomas results in impaired growth and sometimes regression. **a–d,** Transduction of scrambled shRNA served as control. Error bars (**b**, **d**) indicate s.e.m. **b–d,** $n = 12$ transplants (**b**, **c**) or tumours (**d**). $^*P \leq 0.05$ and $^{**}P \leq 0.01$ indicate statistical significance of the observed differences.

identify genes that preferentially affect cancer cells but not their normal counterparts. Although promising in concept, assay conditions and cell-line histories can profoundly affect genes identified in these screens[29,30]. Although xenogeneic transplantations of transduced human cells offer improvements[31], they often incompletely simulate carcinoma ontogeny, which depends upon complex interactions with local and systemic environments. By targeting cells in their normal physiological context, we correct these deficits and abrogate many caveats, including epigenetic, genetic and stress-induced alterations in gene expression, all of which introduce heterogeneity and increase coverage requirements when cells are grown on plastic.

Our study accentuates a particular importance of β-catenin in promoting oncogenic effects, as it surfaced at the top of >15,000 genes in our screen. Moreover, increased Hras–mitogen-activated protein kinase signalling drove β-catenin's effects from negative to positive, as normal epidermal growth was actually impeded by β-catenin. In this regard, it is interesting that leukaemias also seem to be more sensitive to activated β-catenin than their normal counterparts[32]. Our findings further suggest that β-catenin's ability to balance tissue growth is exerted through its antagonistic functions in intercellular adhesion and transcriptional activation.

A myriad of new candidates from our screen await further investigation. Among them are chromatin modifiers, which have been increasingly implicated in human cancers[33]. In this regard, our validation of Mllt6 is intriguing, as MLL proteins are known to associate with DOT1L H3K79-methyltransferase complexes[34]. Given Mllt6's selective effects on oncogenic growth, it is tempting to speculate that this protein might function by guiding its histone modifier complex to a key cancer target gene(s). Although detailed understanding of this and other candidates awaits experimentation, our methodology paves the way for future studies aimed at uncovering mechanisms of SCC progression, with the hope of identifying targets that selectively compromise growth of one of the world's most prevalent and life-threatening cancers.

METHODS SUMMARY

Animals were on a C57BL/6 background. Lentiviral production and ultrasound-guided injection into E9.5 amniotic space are as described[1,35]. Transduced embryos were developed to E18.5, after which epidermal suspensions were prepared for gDNA isolation. gDNAs from sets of 30 transduced embryos were combined and used as template for a 21-cycle pre-amplification PCR. For identification and quantification of shRNAs, clean pre-amplification product was sequenced using Illumina HiSeq2000, and the sequencing output was aligned to the TRC 2.x library with Burrows–Wheeler Aligner (BWA) with a maximum edit distance of three. Bioinformatics analyses of RNA-seq data and candidates identified by our screens were performed using IPA software (Ingenuity Systems). Figures were prepared using Adobe Photoshop and Illustrator CS5. Graphing and statistical analyses were performed using Prism 5 (GraphPad Software). Descriptions of antibodies and mouse strains are provided in Methods.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Information Raw RNAseq data can be accessed at Gene Expression Omnibus under accession number GSE48480 and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to E.F. (fuchslb@rockefeller.edu).

LETTER

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Stimulated X-ray emission for materials science

M. Beye[1], S. Schreck[1,2], F. Sorgenfrei[1,3], C. Trabant[1,2,4], N. Pontius[1], C. Schüßler-Langeheine[1], W. Wurth[3] & A. Föhlisch[1,2]

Resonant inelastic X-ray scattering and X-ray emission spectroscopy can be used to probe the energy and dispersion of the elementary low-energy excitations that govern functionality in matter: vibronic, charge, spin and orbital excitations[1–7]. A key drawback of resonant inelastic X-ray scattering has been the need for high photon densities to compensate for fluorescence yields of less than a per cent for soft X-rays[8]. Sample damage from the dominant nonradiative decays thus limits the materials to which such techniques can be applied and the spectral resolution that can be obtained. A means of improving the yield is therefore highly desirable. Here we demonstrate stimulated X-ray emission for crystalline silicon at photon densities that are easily achievable with free-electron lasers[9]. The stimulated radiative decay of core excited species at the expense of non-radiative processes reduces sample damage and permits narrow-bandwidth detection in the directed beam of stimulated radiation. We deduce how stimulated X-ray emission can be enhanced by several orders of magnitude to provide, with high yield and reduced sample damage, a superior probe for low-energy excitations and their dispersion in matter. This is the first step to bringing nonlinear X-ray physics in the condensed phase from theory[10–16] to application.

In the soft X-ray region, the use of nonlinear techniques to enhance the signal levels has been prevented by the small cross-sections and the short lifetimes of core-excited states in the regime of a few femtoseconds. In the past few years, free-electron lasers have become available, producing ultrashort, intense soft X-ray pulses[9,17–20]. Recently, the stimulation of emission from a single fluorescence line in a rare gas[21] and hard X-ray/optical sum frequency generation[22] have been demonstrated.

We present here stimulated X-ray emission from a solid-state sample recorded at the free-electron laser in Hamburg (FLASH) for non-resonant silicon *L*-edge excitation at a photon energy of 115 eV. With free-electron laser radiation we produce regions with high 2*p* core excitation densities. The spontaneously emitted radiation from recombination of the 2*p* core holes (photon energy of 85 eV to nearly 100 eV) seeds the stimulated emission of soft X-ray photons. The emitted spectrum is determined by the spontaneous emission as observed in a typical resonant inelastic X-ray scattering (RIXS) or X-ray emission spectroscopy (XES) experiment and thus conserves all the information and specificity of these methods. By carefully choosing the geometry, we can significantly enhance the weak fluorescence signal at the expense of Auger decays. Fewer electrons are emitted and electronic damage to the sample is minimized. By properly shaping the free-electron laser beam footprint on the sample the detected signal can be enhanced by orders of magnitude because the usually isotropic emission can be directed towards the detector. This opens up the possibility of nonlinear spectroscopy in the X-ray region by combining concepts from nonlinear optics with the high information depth of X-ray spectroscopy, which is intrinsically able to resolve femtosecond dynamics[10–16,23–25].

The absorption and stimulated emission probabilities *P* can be approximated for infinitesimally thin samples where the photons interact on a short length d*x* as:

$$P = \sigma\rho_{\mathrm{atom}}\,\mathrm{d}x \text{ and } P = \sigma\rho_{\mathrm{ch}}\,\mathrm{d}x \qquad (1)$$

where ρ_{atom} is the number density of absorbing atoms and ρ_{ch} (for core hole) is the number density of excited atoms for stimulation. The X-ray absorption or stimulation cross-sections σ are determined by the dipole transition matrix elements between the core level and unoccupied (absorption) or occupied (stimulated emission) valence states and are assumed at this point to be approximately the same. The cross-section is connected with the absorption length $\lambda = (\sigma\rho_{\mathrm{atom}})^{-1}$. The effect of stimulated emission is large, when the stimulation probability approaches unity $P \overset{!}{=} 1 = \sigma\rho_{\mathrm{ch}}\,\mathrm{d}x$, yielding:

$$\frac{\rho_{\mathrm{ch}}}{\rho_{\mathrm{atom}}} = \frac{\lambda}{\mathrm{d}x} \qquad (2)$$

For effective stimulation, the absorption length relative to the interaction lengths of stimulating photons has to be similar to the fraction of core-excited atoms. For comparable interaction and absorption lengths, every atom needs to be core-excited; a population inversion is required.

In the forward direction, stimulated X-ray emission has been demonstrated in the gas phase[21]. In this geometry, the maximum interaction length at which stimulating photons can interact with core holes is intrinsically given by the absorption length. The sample has to absorb one X-ray photon per atom. If applied to solids, this energy usually destroys the bonding network and is hence not suited for spectroscopic studies of the undisturbed system.

The situation is very different for other geometries. Along the lateral dimension of the X-ray focus, the interaction length can be significantly larger. With soft X-rays on solids, the absorption length of the exciting radiation with a photon energy that is slightly above an absorption edge is typically tens or hundreds of nanometres, whereas the lateral dimensions of typical X-ray foci are a few to hundreds of micrometres. The interaction length is then limited by the absorption length of the RIXS and XES signals with a photon energy below the absorption edge, which are typically an order of magnitude larger than for the exciting radiation. Consequently, lower excitation densities are needed, when the observation direction is chosen to be along the lateral dimension of the projected focus instead. This situation is sketched in Fig. 1, together with measured data of the total emission signal detected at several angles to the surface. We observe an emission maximum at around 9°, which is the result of the balance between the absorption length of the exciting radiation and the interaction length of the emitted radiation inside the excited volume.

We also studied the emission with a spectrometer. Experimental constraints meant that it was placed at around 15° to the sample surface. We observed a characteristic dependence of the total number and the spectral distribution of the outgoing photons on the incoming photon flux (Figs 2 and 3).

Before we analyse the experimental data, we discuss the relevant processes that have to be considered to derive the number of stimulated photons. The explicit formulation is given in the Methods section. Stimulated emission can be treated along the same lines as absorption. We can hence formulate a differential equation as a function of the interaction length in an ansatz similar to the derivation of the Lambert–Beer law for absorption. This approach is inherently time-independent. Auger

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[1]Institute for Methods and Instrumentation of Synchrotron Radiation Research G-ISRR, Helmholtz-Zentrum Berlin für Materialien und Energie GmbH, Albert-Einstein-Straße 15, 12489 Berlin, Germany. [2]Fakultät für Physik und Astronomie, Universität Potsdam, Karl-Liebknecht-Straße 24–25, 14476 Potsdam, Germany. [3]Institut für Experimentalphysik, Universität Hamburg and Centre for Free-Electron Laser Science, Luruper Chaussee 149, 22761 Hamburg, Germany. [4]II. Physikalisches Institut, Universität zu Köln, Zülpicher Straße 77, 50937 Köln, Germany.

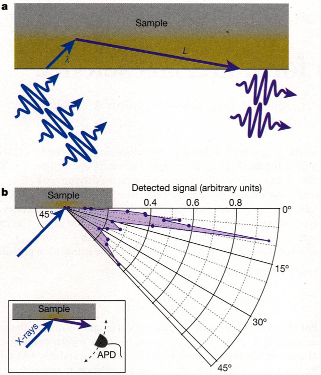

Figure 1 | Geometry to observe spontaneously stimulated X-ray emission from solids. a, X-rays create core excitations in the solid (yellow). A cascade of stimulated emission builds up in a direction where the penetration depth λ of incoming photons is balanced by the absorption length L for emission. **b,** The total emission is detected as a function of glancing angle at fluences where stimulated emission saturates. An enhancement is observed for shallow angles, where the interaction length for emitted photons is longest. This direction is far away from the specular reflection increase around 45°. The inset displays the layout of the experiment. APD, avalanche photodiode.

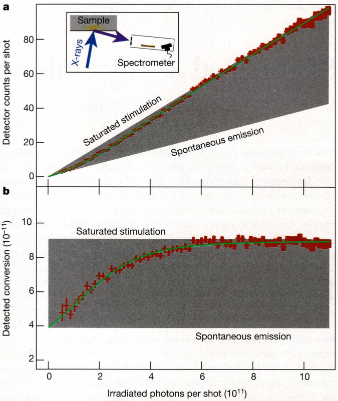

Figure 2 | Observing stimulated emission from a solid. a, Red symbols show the measured number of counts per shot versus the number of incoming photons. The error bars are one standard deviation of the averaged values. The grey area falls between the limiting cases of spontaneous emission and saturation of stimulated emission. The green line is a fit to the derived formula. The inset shows the geometry of the experiment. **b,** The same data are plotted as detected conversion, after dividing the detector counts by the number of incoming photons. The linear limits now become constants.

decays are not explicitly treated, because Auger processes and radiative emission are independent processes and are only related through the number of core holes. Auger decays are implicitly included, though, through the core-hole life time. Auger decays are the only other significant decay channel of the core holes and stimulated emission reduces the number of core holes, so we expect a substantial decrease in the number of Auger processes. The effective lifetime of the core holes, taking all decay channels into account, should be shortened.

We formulate a differential equation for the gain in the number of observed stimulated photons. $N_{\mathrm{stim,obs}}$ is the number of stimulated photons that are emitted into a detector with a finite acceptance angle and detection efficiency:

$$\frac{\mathrm{d}N_{\mathrm{stim,obs}}}{\mathrm{d}x}=N_{\mathrm{obs}}\rho_{\mathrm{ch}}\sigma_{\mathrm{stim}} \qquad (3)$$

where the cross-section for transitions between the respective valence and the core state is σ_{stim} (now generally different from σ_{abs}). The total number of photons in the observation direction N_{obs} drives the observed stimulation and is the sum of the number of spontaneously emitted photons in this direction $N_{\mathrm{sp,obs}}$ (initially acting as a seed) and the increasing contribution of $N_{\mathrm{stim,obs}}$ itself. Without stimulation, $N_{\mathrm{sp,obs}}$ is proportional to the number of the incoming photons. Stimulated photons are available to stimulate further, which leads to a nonlinear increase of the stimulated signal. At low photon numbers stimulation is negligible and the spontaneous emission forms a linear lower limit for the dependence of the observed number of photons as a function of incoming photon numbers.

The next important factor is the instantaneous density of the core holes available for stimulated decays. Although every photon in an X-ray pulse is absorbed in the sample, the instantaneous number of core holes is smaller than one per incoming photon, depending on the pulse length and the core-hole lifetime: some core holes have already decayed before the pulse is over. Besides the reduction through spontaneous decay, the number of core holes is reduced by each stimulated photon. To treat this effect, we split the decay rate of the core holes into two parts: the Auger decays enter through the fixed core-hole lifetime, and the stimulation directly reduces the number of core holes. Thus, the change in the number of stimulated photons depends on $N_{\mathrm{stim,obs}}$ itself, this time with a negative sign leading to saturation. An upper limit for stimulated emission is reached, at which every core hole is stimulated. For high incoming photon numbers, the observed number of photons will thus saturate towards an upper linear limit as a function of incoming photon numbers.

The solution of the differential equation provides the dependence of the observed number of stimulated photons on the experimental parameters. We approximate the lateral distribution of the incoming beam and the exponentially decaying absorption profile into the depth of the sample with a constant core-hole density (albeit modified through stimulated emission), inside a cylindrically shaped excitation volume, the size of which is given by the focal sizes and the absorption length of the incoming radiation. The interaction length for stimulation in this volume extends from the bottom of the excited cylinder to the sample surface in the direction of observation and is thus given only through the geometry of the experiment.

For soft X-rays from current free-electron lasers, focal sizes of the order of 10 μm and pulse lengths around the core-hole lifetime are well within reach. With typical absorption lengths in solids for the incoming photons of around 100 nm and for the emission of around 1 μm and with stimulation cross-sections of 1 Mbarn (ref. 26), stimulation

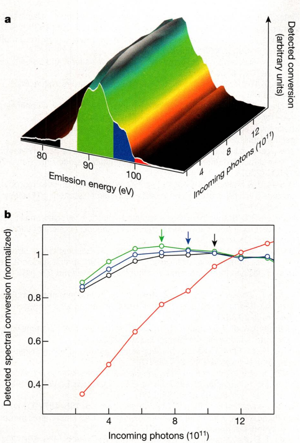

Figure 3 | Spectrally resolved stimulated emission. a, The detected conversion is shown, highlighting the integration regions for data plotted in **b. b,** The maximum detected conversions for different spectral regions are marked with arrows. The most intense features saturate first (green, 87–95 eV), followed by the emission shoulder (blue, 95–99.5 eV) and the weak multiple scattering background (black, 71–84 eV). The onset of saturation can be connected with the stimulation cross-section. Emission above the band gap (red, 99.5–108 eV) is not observed at low intensities. The signal is connected with X-ray-induced electronic excitations.

becomes important at about 10^{11} incoming photons per pulse. Typical soft X-ray free-electron lasers produce up to 10^{13} photons per shot, so that stimulated emission has to be considered as a dominating effect.

We now turn to the experimental observations. In Fig. 1b, we show the angular dependence of the emission signal. The incidence angle is chosen to be 45° and we find enhanced intensity for the specular geometry with the detector at 45°. Additionally, we find a strong enhancement of the signal by about a factor of five at grazing angles around 9°, far from the specular reflection. It appears in a narrow angular window and is indeed the result of a nonlinear effect. With the used excitation conditions, we are deep in the regime where the stimulated emission saturates and the observed effects are variations in the angular distribution of the stimulated emission. The maximum signal is expected in a direction for which the interaction length inside the core excited volume is as large as possible and for which the reabsorption probability is minimized. Because the absorption length for the incoming radiation is about an order of magnitude shorter than the reabsorption length, the optimal angle is expected around $\arcsin(0.1) \approx 6°$ which is in reasonably good agreement with the experiment.

In Fig. 2a, we show the total number of observed photons in our spectrometer placed at 15° to the surface plane, along with a fit to the expected dependence. We also include the linear limits derived from the fit. We observe that already in the lower fluence range studied, there is a deviation from linearity and the stimulated emission quickly saturates to the upper limit. In the chosen experimental geometry, the enhancement of the emission between purely spontaneous and saturated stimulation is only about a factor of two. This is because the majority of the stimulated photons are radiated more grazing to the surface and do not reach our detector, as shown in Fig. 1b. Still, the nonlinear enhancement and saturation of the signal through stimulated emission are clearly observed.

It is instructive to display the total number of observed photons divided by the number of incoming photons (see Fig. 2b). This yields the conversion efficiency from incoming photons to emitted photons, as seen by the detector—the detected conversion. The limiting linear dependences turn into limiting constant conversion efficiencies and are determined by the experimental parameters. The nonlinearity of the curve becomes obvious as a monotonous change from the lower to the upper limit.

The photon number needed for the nonlinear part between the limiting lines is directly related also to the emission-energy-dependent stimulation cross-section. For larger stimulation cross-sections, the nonlinearity and thus the saturation of stimulated emission will occur at smaller incoming photon numbers. In Fig. 3a, we show the spectral evolution of the silicon *L*-edge emission depending on the number of incoming photons and how we separate the spectrum into parts of different emission intensity. The stimulation cross-section contains the same matrix elements as the spontaneous emission probability, which is in turn proportional to the measured spectral emission intensity, so the stimulation of emission becomes more effective for emission-energy regions that already show a high intensity at low fluences. Figure 3b displays the dependence of the normalized detected conversion on the number of incoming photons in specific emission-energy regions. The peak region around an emission energy of 90 eV, as the most intense feature, approaches saturated stimulation fastest, and the other regions follow in the order of spectral intensity.

The emission energy region for usually unoccupied states above the bandgap shows a strikingly different behaviour. Here, a secondary effect sets in. Photo-emitted electrons and electrons from Auger decays scatter inelastically in the sample, very quickly creating a multitude of delocalized electron–hole pair excitations around the bandgap. This effect becomes stronger with an increasing number of incoming photons and the connected radiative decays annihilate core holes that contributed to the stimulated emission of other parts of the spectrum at lower photon numbers. Therefore, the detected conversion in other parts of the spectrum actually shrinks rather than becoming constant. Nevertheless, because all emission processes saturate when all the core holes are stimulated down, the total detected conversion still becomes constant (compare the integral detected conversion shown in Fig. 2b).

In the direction of 15° from the surface, the detected conversion increases by a bit more than a factor of two between the limiting cases of spontaneous emission and saturated stimulated emission. By using a shallower angle, we can increase the gain further by another factor of five (see the difference in the saturated emission signal in Fig. 1b). This value is limited by the round shape of the free-electron laser beam footprint on the sample. The shape of the beam leads to the same maximized interaction length in every azimuthal direction in the sample surface plane and thus to a stimulated emission profile which is rotationally symmetric around the sample normal at the centre of the beam.

The observed gain can clearly be further increased when the footprint of the irradiated photons is specifically shaped. An elongation of the footprint in the observation direction will further enhance the observed photon numbers, whereas in other directions the signal will be reduced. To ensure temporal overlap of the core excitations in the sample and the majority of the stimulated photons travelling with the speed of light, the footprint of the exciting beam needs to be elongated by placing the sample grazing to the incident beam. In this way, the exciting wavefront will travel along the sample surface in time with the stimulated photons and the enhancement of the emission signal will be maximized while X-ray-induced sample damage is further minimized.

The same applies to soft X-ray experiments from other systems, because the determining parameters are very similar for most materials.

The signal gain that we expect as compared to normal RIXS or XES experiments stems from two sources. On one hand, the saturation of stimulated emission is an indicator that most core holes decay via the emission of photons instead of decaying via Auger processes. This pushes the observed fluorescence yield from the 10^{-3} level in the direction of unity. On the other hand, directing the majority of the emission towards the detector can substantially increase the signal, given that typical fluorescence detectors (and our spectrometer) have a geometrical acceptance of less than 10^{-4} of the full solid angle. Therefore, stimulated emission can enhance the detected signal by several orders of magnitude. In our experiment we did observe traces of sample damage. Generally though, with optimized geometries at optimized X-ray sources, the signal gain can be used to minimize acquisition times in the non-damaging regime, or alternatively to record full spectra in a single X-ray shot, so that the spectroscopic information is essentially generated before X-ray-induced changes set in.

Such enhanced signal levels enable highly selective RIXS and XES studies with considerably improved energy resolution, so that new low-energy excitations may be discovered. Through the combination with pump–probe techniques, huge parameter spaces in the ultrafast time domain can quickly be mapped. Finally, through the combination of nonlinear techniques from optical lasers with the high specificity of X-rays, small signals from extremely dilute active centres can now be dissected.

METHODS SUMMARY

Photon parameters. The experiments were conducted at the free-electron laser FLASH at Hamburg, Germany, operating at a photon energy of 115 eV. The spectrally resolved data was recorded at a repetition rate of 5 Hz with 30-fs pulses[17,27]. We used the grating of beamline PG 2 as a mirror[28]. The focus was round with a diameter of 45 µm, leading to fluences up to 1 J cm^{-2}. The angular dependence was measured at beamline BL 2 (ref. 29) at a burst repetition rate of 10 Hz, each including 100 pulses at 250 kHz. The focal size was adjusted to be about 40 µm to compensate for the now 50-fs-long pulses. With a gas attenuator, we set the fluence around 1 J cm^{-2}. The incoming photon numbers have been measured for each shot by a gas monitor detector[29].

Experimental setup. We scanned hydrogen-passivated silicon (100) surfaces through the beam. Spectra were recorded with a Scienta XES 355 spectrometer[30] and a single-photon-counting, multi-hit-capable detector centred at 92 eV with a resolution of 0.4 eV. The scattering plane was the horizontal plane of polarization of the incoming photons with the spectrometer at around 85° to the beam (15° to the sample surface), while the pulses impinged at an angle of 80° to the surface. For the angular dependence, we used an avalanche photodiode with an aluminium filter to block optical light. The incidence angle was 45°, whereas the detection angle was varied.

Analysis. With our spectrometer, we recorded around 40,000 single shots. The data was sorted, binned and the standard deviations of the averaged values were computed for each bin. With the photodiode, we analysed 15,000 bursts. During free-electron laser irradiation, the emission from a plasma plume was optically visible at the sample and slight ablation was observed.

Full Methods and any associated references are available in the online version of the paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.B. (martin.beye@helmholtz-berlin.de).

LETTER

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Stereoinversion of tertiary alcohols to tertiary-alkyl isonitriles and amines

Sergey V. Pronin[1], Christopher A. Reiher[1] & Ryan A. Shenvi[1]

The S_N2 reaction (bimolecular nucleophilic substitution) is a well-known chemical transformation that can be used to join two smaller molecules together into a larger molecule or to exchange one functional group for another. The S_N2 reaction proceeds in a very predictable manner: substitution occurs with inversion of stereochemistry, resulting from the 'backside attack' of the electrophilic carbon by the nucleophile. A significant limitation of the S_N2 reaction is its intolerance for tertiary carbon atoms: whereas primary and secondary alcohols are viable precursor substrates, tertiary alcohols and their derivatives usually either fail to react or produce stereochemical mixtures of products[1–3]. Here we report the stereochemical inversion of chiral tertiary alcohols with a nitrogenous nucleophile facilitated by a Lewis-acid-catalysed solvolysis. The method is chemoselective against secondary and primary alcohols, thereby complementing the selectivity of the S_N2 reaction. Furthermore, this method for carbon–nitrogen bond formation mimics a putative biosynthetic step in the synthesis of marine terpenoids[4] and enables their preparation from the corresponding terrestrial terpenes. We expect that the general attributes of the methodology will allow chiral tertiary alcohols to be considered viable substrates for stereoinversion reactions.

The incorporation of nitrogen during secondary metabolite biosynthesis occurs almost exclusively through the chemistry of carbonyls and imines: reductive amination, Mannich reactions and transamination[5]. The ability to mimic these biosynthetic pathways by chemical synthesis has vastly simplified the production of complex alkaloids. However, many alkaloids produced in marine environments do not adhere to the established biosynthetic patterns of terrestrial organisms (see Fig. 1a). For example, the nitrogen atoms in the terpenoid metabolites **1–6** derive from inorganic cyanide by means of reactions that are not well characterized but certainly do not involve carbonyl or imine substrates. These reactions may involve the attack of high energy, unstabilized carbocations by cyanide or a nucleophile derived from cyanide (Fig. 1b)[4]. Indeed, the primary products of these pathways are isonitriles[6].

Mimicry of this proposed biosynthetic transformation is challenging because, apart from substrate control[7], intermolecular attack of unstabilized carbocations by cyanide or organonitriles is non-stereoselective. Furthermore, the high acidity of protons adjacent to carbocations facilitates E1 (unimolecular) elimination with most counteranions[8], which can erase adjacent stereogenic centres. Therefore, even if reprotonation of the nascent alkene is possible with Brønsted acid, regioisomers and further stereoisomers can result.

Here we demonstrate a chemoselective and stereoselective method for incorporating cyanide into terpenoid frameworks by the stereoinversion of tertiary alcohols. This method is uniquely competent to generate in concise synthetic sequences the primary pharmacophores of isocyanoterpenes[9], some of which have shown potent antimalarial activity[10]. The method is also generally useful for the conversion of alcohols to amines and is chemoselective for tertiary alcohol displacement in preference to secondary or primary alcohols. Previous approaches to the synthesis of terpenoids exemplified by Fig. 1 have relied on either abiotic strategies for formation of the C–N bond[11] or biomimetic reactions of nitriles with carbocations that rely on stereocontrol imparted by a cyclic scaffold[12]. This latter strategy has proved to be capricious, because many terpenoid cations lead to isomeric mixtures or non-natural stereoisomers on capturing nitriles[13–15]. Previous studies did, however, establish the idea of using a Lewis acidic reagent to ionize a tertiary leaving group in the presence of trimethylsilyl cyanide (TMSCN) as a competent nucleophile and isonitrile precursor. For instance, tertiary halides will react with TMSCN in the presence of $TiCl_4$ to produce isonitriles[16]. Similar reactions have simplified the synthesis of a marine bisisonitrile; however, the late-stage isocyanation resulted in a near equimolar mixture of four stereoisomers[13]. Subsequently the ionization of tertiary alcohols with superstoichiometric zinc halides in the presence of TMSCN was developed[17]. However, this latter method is neither stereoselective nor chemoselective and results in indiscriminate isocyanation, elimination and isomerization of, for example, α-bisabolol **10** (Fig. 2a). A stereocontrolled Mitsunobu isocyanation of tertiary alcohols was also reported[18], but in our hands this reaction could not be successfully applied to the isocyanoterpenes[9].

We believed that a stereocontrolled displacement of alcohols or esters could be effected if TMSCN were used in excess and could therefore stereoselectively trap the nascent cation at the contact ion-pair stage[1,20].

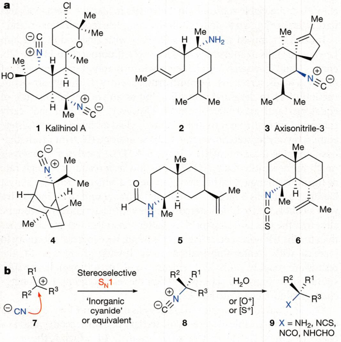

Figure 1 | Nitrogenous marine terpenoids derived from inorganic cyanide. **a**, A subfamily of marine terpenoids represented by **1–6** are decorated with nitrogen atoms in a different pattern from that observed in most terrestrially derived metabolites. **b**, From isotope-feeding studies the hypothetical biosynthetic path (**7**→**8**→**9**) common to **1–6** is known to incorporate inorganic cyanide and is proposed to involve unstabilized carbocations as biosynthetic intermediates.

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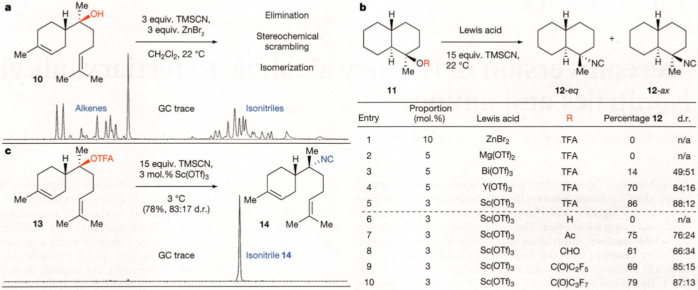
[1] Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA.



Entry	Proportion (mol.%)	Lewis acid	R	Percentage **12**	d.r.
1	10	ZnBr$_2$	TFA	0	n/a
2	5	Mg(OTf)$_2$	TFA	0	n/a
3	5	Bi(OTf)$_3$	TFA	14	49:51
4	5	Y(OTf)$_3$	TFA	70	84:16
5	3	Sc(OTf)$_3$	TFA	86	88:12
6	3	Sc(OTf)$_3$	H	0	n/a
7	3	Sc(OTf)$_3$	Ac	75	76:24
8	3	Sc(OTf)$_3$	CHO	61	66:34
9	3	Sc(OTf)$_3$	C(O)C$_2$F$_5$	69	85:15
10	3	Sc(OTf)$_3$	C(O)C$_3$F$_7$	79	87:13

Figure 2 | Development of a stereoselective Ritter-type reaction through tertiary alcohol inversion. a, Prior state-of-the-art isocyanation of tertiary alcohols applied to α-bisabolol **10** produces complex mixtures. GC, gas chromatography. **b,** Optimization studies identify Sc(OTf)$_3$ as an optimal Lewis acid and fluoroesters as optimal substrates for stereoinverting isocyanation. Eq, equatorial; ax, axial; d.r., diastereomeric ratio; n/a, not applicable. **c,** Application of this method to bisabolyl trifluoroacetate **13** provides isonitrile **14** cleanly and with high stereoselectivity.

The challenge of this approach lies in the competitive equilibria of Lewis acid-catalysed solvolysis. First, the Lewis-basic TMSCN and the Lewis-basic leaving group compete for association with the requisite Lewis acid. In the extreme case of solvolysis, the Lewis acid might be coordinatively saturated with non-labile solvent and rendered unreactive. Second, the counteranion that is necessary for steric and/or electronic shielding of one face of the carbocation can undergo equilibrium displacement with TMSCN[20], which would result in a stereoisomeric mixture of solvolysis products. Unfortunately, whereas chiral tertiary chlorides[21] are viable substrates for solvolysis in alkaline methanol[1], tertiary esters are known to undergo almost complete racemization in acid[2].

Indeed, attempted solvolysis in TMSCN with zinc and magnesium Lewis acids fails to consume any decalinyl trifluoroacetate **11** (Fig. 2b, entries 1 and 2; see also Supplementary Information for a complete list of Lewis acids screened). Bismuth triflate (entry 3) shows slow conversion of **11** to decalinyl isonitriles, but as an equimolar mixture of equatorial and axial stereoisomers. However, we found that early transition metal triflates (entries 4 and 5) yield a high ratio of diastereomers favouring stereoinversion (**12**). Scandium(III) trifluoromethanesulphonate (scandium(III) triflate; Sc(OTf)$_3$) catalyses[22] solvolysis much more rapidly than Y(OTf)$_3$, possibly as a result of its high rate of ligand exchange[23]; it therefore can be conveniently run at catalyst loadings as low as 3 mol%. The solvolysis was found to be ineffective with alcohols, and to be less stereoselective with acetyl and formyl esters, although the reaction rate for these latter substrates was much higher than for the trifluoroacetate. Given this trend, we expected selectivity to increase with greater fluorination of the leaving group. For **11**, the results are roughly equivalent (entries 9 and 10), although linear substrates show improved selectivity when the leaving group is a longer-chain perfluoroalkanoate (see below).

The optimal conditions for the solvolysis of decalin **11** were found to be generally applicable to stereogenic tertiary trifluoroacetates, and as a result **13** can be cleanly converted to isonitrile **14**, which is the enantiomer of a naturally occurring marine terpenoid (Fig. 2c)[4].

The generality, functional group tolerance and utility of this solvolysis reaction are illustrated by Fig. 3. When a tertiary alcohol is the sole stereocentre in a molecule, the percentage inversion of **15**, for instance, is 90%, accounting for enantiomeric purity of the alcohol. It should be noted that acyclic perfluorobutyrates showed slightly higher stereoselectivity in solvolysis (at −5 °C with 10% (v/v) CH$_2$Cl$_2$) than the corresponding trifluoroacetates: **14** (diastereomeric ratio 85:15) and **15** (enantiomeric ratio 87:13, corresponding to 93% inversion). Proximal unsaturation (for example **16**) has little or no effect on stereoselectivity. The diastereomeric ratio of isonitriles **17** is almost identical to the percentage inversion of **15**, and so the distal stereocentre seems to have essentially no influence but serves as a useful stereochemical probe. Lewis basic groups such as esters (**16**) and nitriles (**17**) are tolerated but tend to decrease reaction rates; so catalyst loading was therefore increased to 6 mol%. Not surprisingly, alkynes **20** are tolerated, as are primary alcohols **21**, which are also subjected to solvolysis conditions as their corresponding trifluoroacetates but do not ionize.

Naturally occurring marine terpenes are easily accessible with this method (also see Fig. 4). For instance, isocyanocadinene[4] **22** is synthesized in two steps from its terrestrial counterpart, cedrelanol (seven steps from commercial material), whereas **22** was previously prepared in 29 steps[24]. Additionally, *trans*-androsterone can be elaborated to the corresponding tris-trifluoroacetate (see Supplementary Information), which on solvolysis selectively produces isonitrile **23** with excellent stereoselectivity and chemoselectivity. This stereoinversion reaction shows the same stereochemical preference as an S$_N$2 reaction, but with reverse substitution demands. Tocopherol-derived quinone **24** can be accessed with high diastereoselectivity, obviously independently of its two other stereocentres. The monoisonitrile **25** and bisisonitrile **26** can be derived from dihydrosclareol by means of one and two stereoinversions, in which selectivity presumably reflects the different heterolytic stabilities of the two trifluoroacetates.

This procedure enables the stereoinversion of tertiary alcohols to the corresponding tertiary-alkyl amines and their derivatives (Fig. 4). For instance, 1.11 g of α-bisabolol (**10**) can be trifluoroacetylated and solvolysed to isonitrile **14**, which in turn can be hydrolysed to 1.10 g of amine **2**, which was isolated as a 5:1 ratio of diastereomers. The enantiomer of **27** was previously synthesized in nine steps (19 mg)[25]. Simple hydrolysis also converts androsterone-derived isonitrile **23** into amine **30**, and quinone **24** into aza-tocopherol **31** (after reduction of the intermediate iminoquinone). Naturally occurring cadinenes **28** and **29** (ref. 4) are simple to access from **22** by means of hydrolysis or sulphurization (see Supplementary Information).

This isocyanation is of course not without limitation (Fig. 5). Highly branched tertiary alcohols (**31–33**) generally give poor diastereoselectivity, probably because of an encumbered approach of the nucleophile.

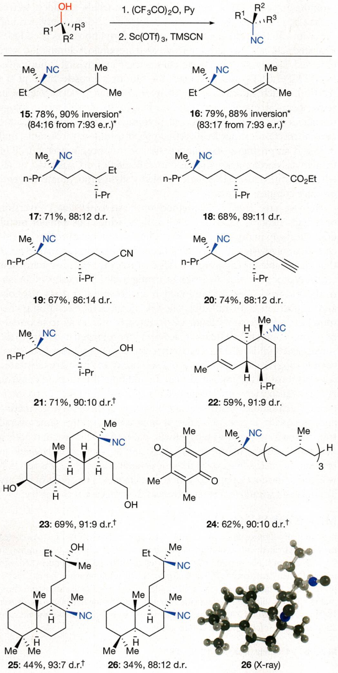

OH
R1
R3
R2
1. (CF3CO)2O, Py
2. Sc(OTf)3, TMSCN
R1
R2
R3
NC
15: 78%, 90% inversion*
(84:16 from 7:93 e.r.)*
16: 79%, 88% inversion*
(83:17 from 7:93 e.r.)*
17: 71%, 88:12 d.r.
18: 68%, 89:11 d.r.
19: 67%, 86:14 d.r.
20: 74%, 88:12 d.r.
21: 71%, 90:10 d.r.†
22: 59%, 91:9 d.r.
23: 69%, 91:9 d.r.†
24: 62%, 90:10 d.r.†
25: 44%, 93:7 d.r.†
26: 34%, 88:12 d.r.
26 (X-ray)

Figure 3 | Probing the selectivity and functional group tolerance of isocyanation. Py, pyridine; e.r., enantiomeric ratio; d.r., diastereomeric ratio. *Determined by Mosher analysis. †After treatment with methanol and triethylamine.

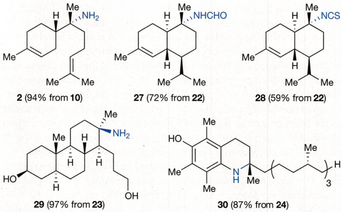

NH2
NHCHO
NCS
2 (94% from 10)
27 (72% from 22)
28 (59% from 22)
NH2
29 (97% from 23)
30 (87% from 24)

Figure 4 | Amines, amides and isothiocyanates synthesized from the corresponding isonitriles. Chemoselective and stereoselective isocyanation allows access to a variety of marine metabolites and allows terrestrial terpenoids to be converted to their theoretical marine counterparts.

Furthermore, we discovered early on that cyclohexanols are problematic substrates. Whereas equatorial trifluoroacetate **34** is solvolysed to the axial isonitrile **36** with high stereoselectivity, the displacement of axial trifluoroacetate **35** is slower and poorly diastereoselective, and actually favours stereoretention. We suspect that this low stereoselectivity is due to the imperfection of the 4-*t*-butyl conformational 'lock'[26] that allows enough torsional mobility for ionization at the 1 position to proceed preferentially though a twist-boat conformer[27]. The exact relationship between torsional freedom and stereoselection is not clear, but nevertheless the more rigid decalins **37** and **11** do not suffer from the same promiscuity. It remains unclear whether C–N bond formation occurs at the contact ion pairs of equatorial esters **34** and **37**, because solvent-separated cations of **34** and **37** yield the same axial isonitrile products[9]. Nevertheless, we are optimistic that these solvolysis conditions may help to shed light on the still obscure stereoelectronic basis for stereoselective addition to cyclohexyl cations[28].

A detailed mechanistic picture of the solvolysis (for example, **13→14**) is not yet available, but a general sketch (Fig. 6) and relevant observations are worth discussion. Because Sc(OTf)$_3$ is a strong Lewis acid, we suspect that it becomes coordinatively saturated (**A**) on addition to TMSCN, and we observe that it dissolves completely. By analogy with the high rate constant for inner-sphere water ligand exchange of Sc(OTf)$_3$ observed previously[23], we suspect that **A** rapidly exchanges a TMSCN ligand for ester **B** to provide activated substrate **C**. Two reactions are then available to **C**: either S_N2 or S_N1 displacement (path

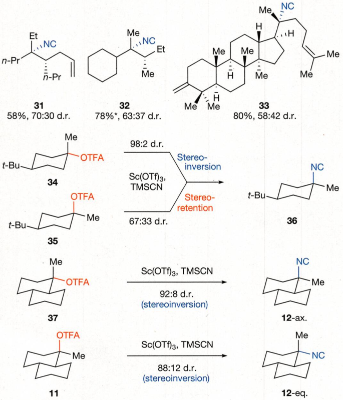

31
58%, 70:30 d.r.
32
78%*, 63:37 d.r.
33
80%, 58:42 d.r.
34
35
98:2 d.r.
Sc(OTf)3, TMSCN
67:33 d.r.
Stereo-inversion
Stereo-retention
36
37
Sc(OTf)3, TMSCN
92:8 d.r.
(stereoinversion)
12-ax.
11
Sc(OTf)3, TMSCN
88:12 d.r.
(stereoinversion)
12-eq.
OTFA
NC

Figure 5 | Limitations of tertiary alcohol stereoinversion. Highly branched substrates provide lower diastereomeric ratios of isonitriles, as do conformationally flexible cyclohexanol derivatives. OTFA, trifluoroacetate. *Isolated along with regioisomeric isonitriles.

1 or path 2). It is possible that departure of the ester and attack by TMSCN occur concurrently (path 1), in which the nucleophile stabilizes the developing positive charge by means of nascent bond formation (**D**), but this occurrence is rare[3] and has been ruled out in related solvolysis reactions[19]. An alternative possibility is that ionization of **C** occurs (path 2) to provide contact ion pair **E**, and TMSCN approaches the planar carbocation opposite the large and electron-rich counter-anion to provide stereochemically inverted isonitrile **8** after silyl transfer to the trifluoroacetate. If instead of attack **E** undergoes ion dissociation to solvated cation **F** (path 3), then attack of **F** from either face would provide the stereochemically scrambled isonitrile **rac-8**. Thus, the stereochemical ratio of product isonitriles should depend on the relative rates of attack (**E**→**8**) versus anion–solvent exchange (**E**→**F**), and two observations are worth noting. First, the identity of the ester (R = H, CH_3, CF_3, C_3F_7) affects the stereochemical outcome, but the contribution of these esters to the relative rates of attack versus anion–solvent exchange is unclear. Transient interactions between the fluorines on the perfluoroalkanoates and the carbocation[29], or differential distribution of charge between the alkanoate and metal centre[30], could explain this trend. The overall reaction rates of acetates are higher than those of trifluoroacetates, but the inverse relationship of reaction rate and stereochemical outcome may be coincidental. Second, the percentage inversion of trifluoroacetyl ester **13** to isonitrile **14** was found to be inversely proportional to temperature: 79:21 at 22 °C, compared with 83:17 at 3 °C. Given these trends, further investigation is necessary to provide a clear mechanistic picture; however, three control experiments were also performed in support of Fig. 6. Replacement of $Sc(OTf)_3$ with trimethylsilyl triflate (TMSOTf) under otherwise identical conditions did not promote any reaction of trifluoroacetyl esters, which excludes electrophilic silicon as the active Lewis acid. Addition of 2,6-di-*t*-butylpyridine (3:1 with catalyst) did not inhibit product formation, which excludes Brønsted acid catalysis, although increased amounts of elimination were observed. Finally, addition of stoichiometric trimethylsilyl trifluoroacetate ($TMSO_2CCF_3$) did not affect the rate or stereoselectivity of the reaction, which indicates no participation of the product acetate in stereochemical scrambling. We are working to expand this methodology to include other nucleophiles, but preliminary data suggest that the current reaction cannot be directly applied to different reactants, and in fact is surprising in its uniqueness. For instance, in acetonitrile or trimethylsilylacetonitrile, the perfluoroalkanoates do not react at all, whereas trimethylsilylazide and trimethylsilylisothiocyanate solvents produce complex mixtures.

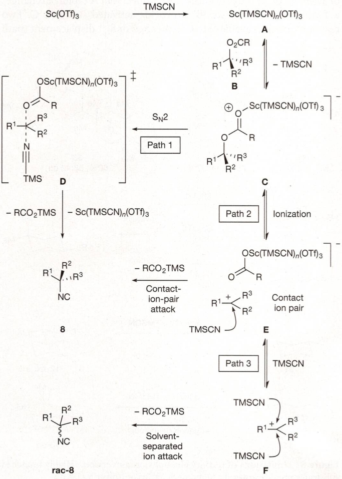


Figure 6 | Possible reaction pathways. A sketch of mechanistic possibilities according to experimental observation.

Here we have developed a method for the stereoselective conversion of tertiary alcohols to tertiary-alkyl amines, which fills a major methodological gap in organic chemistry. The reaction entails a Lewis-acid-catalysed solvolysis of tertiary alcohol derivatives, a conceptual advance that may enable the expansion of this chemistry to related systems. The reaction proceeds with inversion of configuration by means of an isonitrile and works best on minimally branched linear tertiary alcohols, and conformationally inflexible alicyclic alcohols. The reaction is chemoselective for tertiary-trifluoroacetyl esters in preference to secondary or primary ones, which do not react even on prolonged exposure to the solvolysis conditions. This reactivity complements standard stereoinversion (S_N2) reactions, which favour secondary and primary substrates, whereas most tertiary substrates fail. In addition, this method converts readily available terrestrial terpenoid alcohols into their marine counterparts, which should greatly facilitate the preparation of these fascinating and potentially useful molecules. It is possible that the same overall transformation is used in biosynthetic pathways. This reaction adds a new retrosynthetic manoeuvre to the cache of transforms available to organic chemistry. We expect that the general attributes of this reaction will lead to the development of other stereoinversion reactions of tertiary alcohols and stimulate further advances in carbocation chemistry.

METHODS SUMMARY

A solution of trifluoroacetate **13** (32.0 mg, 0.1 mmol) in TMSCN (0.1 ml) was cooled to 0 °C and treated with a solution of anhydrous $Sc(OTf)_3$ (1.5 mg, 0.003 mmol) in TMSCN (0.1 ml). The reaction mixture was left for 18 h at 3 °C and quenched with tetramethylethylenediamine (7.5 μl, 0.05 mmol). The resulting solution was concentrated under reduced pressure, and the residue was purified by flash column chromatography on silica gel (elution with 35% dichloromethane in hexanes) to deliver 18.0 mg (78% yield) of 7-isocyano-7,8-dihydro-α-bisabolene (**14**). See the notes in Supplementary Information regarding safety.

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Supplementary Information is available in the online version of the paper.

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Author Contributions S.V.P. and R.A.S. conceived the work. S.V.P. and C.A.R. performed the experiments. S.V.P., C.A.R. and R.A.S. designed the experiments and analysed the data. R.A.S. wrote the manuscript.

Author Information Crystallographic data are deposited with the Cambridge Crystallographic Data Centre under accession numbers CCDC 949094 (**25**) and CCDC 948968 (**26**). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.A.S. (rshenvi@scripps.edu).

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Changes in North Atlantic nitrogen fixation controlled by ocean circulation

Marietta Straub[1], Daniel M. Sigman[2], Haojia Ren[3], Alfredo Martínez-García[1], A. Nele Meckler[1], Mathis P. Hain[2] & Gerald H. Haug[1]

In the ocean, the chemical forms of nitrogen that are readily available for biological use (known collectively as 'fixed' nitrogen) fuel the global phytoplankton productivity that exports carbon to the deep ocean[1–3]. Accordingly, variation in the oceanic fixed nitrogen reservoir has been proposed as a cause of glacial–interglacial changes in atmospheric carbon dioxide concentration[2,3]. Marine nitrogen fixation, which produces most of the ocean's fixed nitrogen, is thought to be affected by multiple factors, including ocean temperature[4] and the availability of iron[2,3,5] and phosphorus[6]. Here we reconstruct changes in North Atlantic nitrogen fixation over the past 160,000 years from the shell-bound nitrogen isotope ratio ($^{15}N/^{14}N$) of planktonic foraminifera in Caribbean Sea sediments. The observed changes cannot be explained by reconstructed changes in temperature, the supply of (iron-bearing) dust or water column denitrification. We identify a strong, roughly 23,000-year cycle in nitrogen fixation and suggest that it is a response to orbitally driven changes in equatorial Atlantic upwelling[7], which imports 'excess' phosphorus (phosphorus in stoichiometric excess of fixed nitrogen) into the tropical North Atlantic surface[5,6]. In addition, we find that nitrogen fixation was reduced during glacial stages 6 and 4, when North Atlantic Deep Water had shoaled to become glacial North Atlantic intermediate water[8], which isolated the Atlantic thermocline from excess phosphorus-rich mid-depth waters that today enter from the Southern Ocean. Although modern studies have yielded diverse views of the controls on nitrogen fixation[1,2,4,5], our palaeobiogeochemical data suggest that excess phosphorus is the master variable in the North Atlantic Ocean and indicate that the variations in its supply over the most recent glacial cycle were dominated by the response of regional ocean circulation to the orbital cycles.

Nitrogen fixation, the conversion of N_2 to ammonia, by cyanobacteria in surface waters seems to dominate the input of fixed N to the ocean. The main loss of fixed N is biological reduction to N_2 (generalized here as 'denitrification') in marine sediments and in the suboxic zones of the water column in the eastern tropical Pacific Ocean and the Arabian Sea[1]. Nitrogen fixation introduces N with $\delta^{15}N$ of about $-1‰$ ($\delta^{15}N = (^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{reference} - 1$, where the reference is atmospheric N_2), whereas water column denitrification preferentially removes ^{14}N-bearing nitrate[9] (NO_3^-). In total, global ocean denitrification raises the $\delta^{15}N$ of mean ocean nitrate above that of newly fixed N (ref. 10).

Sediment records show N isotopic evidence of reduced water column denitrification during the Last Glacial Maximum (LGM) and other cold phases of the most recent glacial cycle, relative to the current interglacial (the Holocene epoch) and times of intermediate climate states[11] (for example Marine Isotope Stage (MIS) 3, from 57 to 29 kyr ago). Benthic denitrification, because of its weak effect on the $\delta^{15}N$ value of nitrate in the water column[10], has not been directly reconstructed, but it may also have decreased during ice ages because of reduced continental shelf area due to lower sea level[12].

The history of N fixation has been vigorously debated[2,3,13]. As with water column denitrification, N fixation is apparent in the spatial pattern of nitrate $\delta^{15}N$. Against the background of the mean ocean nitrate $\delta^{15}N$ of $\sim 5‰$, N fixation in the euphotic zone followed by the sinking and remineralization of organic matter adds low-$\delta^{15}N$ nitrate to the thermocline, which is observed across the western subtropical and tropical North Atlantic[14]. This low $\delta^{15}N$ value is recorded by the organic N bound within the shell walls of planktonic foraminifera[15,16]. Foraminifera-bound $\delta^{15}N$ (FB-$\delta^{15}N$) from the Caribbean Sea and the Gulf of Mexico indicates that N fixation was reduced during the LGM[15,17].

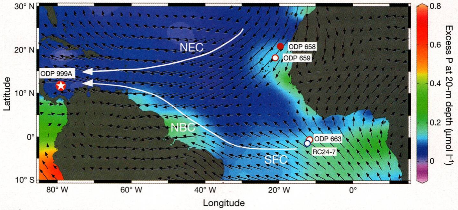

Figure 1 | Core locations, surface winds, excess P at 20-m depth and main surface currents. The star indicates ODP Site 999 Hole A (12° 45′ N, 78° 44′ W; 2,828 m), the core in which FB-$\delta^{15}N$ was measured to reconstruct North Atlantic N fixation. The filled red circle indicates ODP Site 658 (20° 45′ N, 18° 35′ W; 2,263 m), from which sediment Zr/Al data are reported in relation to North African aeolian flux. The open blue circle indicates core RC24-7 (1° 21′ S, 11° 55′ W; 3,899 m), previously published data from which reveal precession cycles in equatorial Atlantic upwelling[7]. The open red circles indicate ODP Site 659 (18° 05′ N, 21° 02′ W; 3,070 m) and ODP Site 663 (1° 11.9′ S, 11° 52.7′ W; 3,708 m), from which come previously published data pertaining to the aeolian flux[21,22]. Excess P ($[PO_4^{3-}] - [NO_3^-]/16$; ref. 6) is an annual average. Black arrows show June–August winds, with length indicating speed. NBC, north Brazil current; NEC, north equatorial current; SEC, south equatorial current.

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[1]Geological Institute, Department of Earth Sciences, ETH Zurich, 8092 Zurich, Switzerland. [2]Department of Geosciences, Princeton University, Princeton, New Jersey 08544, USA. [3]Lamont-Doherty Earth Observatory, Columbia University, New York, New York 10964, USA.

Here we build on the FB-δ^{15}N results of ref. 15 with new data from the same core, Ocean Drilling Program (ODP) Site 999 Hole A in the Caribbean Sea (Fig. 1), extending the record back over the past 160 kyr through a full glacial cycle (Fig. 2a, b). We measured FB-δ^{15}N in two euphotic-zone-dwelling species of planktonic foraminifera, *Globigerinoides ruber* and *Globigerinoides sacculifer*. The records from the two species are very similar (Fig. 2a and Supplementary Fig. 1) and highly coherent (Supplementary Fig. 6). The FB-δ^{15}N of *G. ruber* (Fig. 2a, green symbols) is typically slightly (~0.3‰) lower than that of *G. sacculifer* (Fig. 2a, blue symbols). There is a greater (≥0.6‰) interspecies difference during the LGM, in glacial MIS 4 and during the transition from MIS 6 to MIS 5 (Fig. 2a and Supplementary Fig. 1). Although changes in the interspecies δ^{15}N difference probably hold valuable information[16], given the overarching similarity of the *G. ruber* and *G. sacculifer* FB-δ^{15}N records, we focus hereafter on the FB-δ^{15}N record generated by averaging the two species-specific records (Fig. 2a, black symbols).

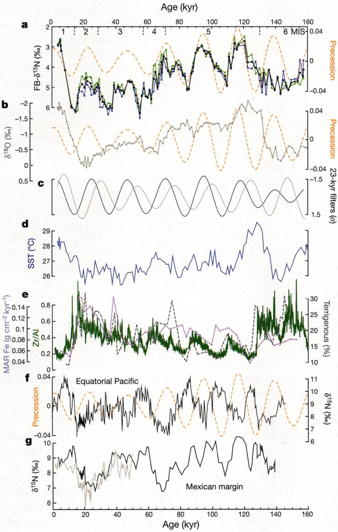

Figure 2 | FB-δ^{15}N in ODP Site 999 and its relationship to SST, dust flux and water column denitrification. a, FB-δ^{15}N was measured separately in two species, *G. sacculifer* (blue) and *G. ruber* (green). The black symbols show the two-species average FB-δ^{15}N at each sampling depth. As plotted, increasing N fixation is upward. **b,** *Globigerinoides ruber* δ^{18}O at ODP Site 999[20]. In **a** and **b**, precession is dashed orange. **c,** The 23-kyr precession-band-filtered FB-δ^{15}N (black) and *G. ruber* δ^{18}O (grey) illustrate the 4–7-kyr phase lag between δ^{15}N and δ^{18}O at the precession band (Supplementary Figs 6 and 9). Given an average phase lag of 4–7 kyr between δ^{18}O and precession[19], this observation confirms the correspondence of δ^{15}N minima with precession maxima (June aphelion). ($\delta^{18}O = ({}^{18}O/{}^{16}O)_{sample}/({}^{18}O/{}^{16}O)_{reference} - 1$, where the reference is Vienna PeeDee Belemnite.) **d,** *Globigerinoides ruber* Mg/Ca-derived SST at ODP Site 999[20]. **e,** Atlantic dust-related records are mass accumulation rate of iron (MAR Fe) at ODP Site 999[23], Zr/Al at ODP Site 658 and terrigenous material abundance at ODP Site 663[22]. **f, g,** The Pacific bulk sediment δ^{15}N records are from the Mexican margin[29] (**g**) and the equatorial Pacific[30] (**f**), with increasing denitrification plotted upward (see also Supplementary Fig. 5).

The FB-δ^{15}N of the previous interglacial (early MIS 5) is similar to that of the Holocene, and the FB-δ^{15}N of the penultimate glacial maximum (MIS 6) is similar to that of the LGM. There is no evidence from previous work for mean ocean nitrate δ^{15}N changes across the most recent glacial cycle that mimic the FB-δ^{15}N record of Site 999, with the exception of a possible δ^{15}N maximum during the most recent deglaciation[15,18]. Furthermore, South China Sea FB-δ^{15}N data support the interpretation of the Caribbean LGM-to-Holocene FB-δ^{15}N decrease as recording a regional change in N fixation[18]. The FB-δ^{15}N change through the Caribbean Sea record is of similar amplitude to the regional isotopic imprint of N fixation, in which nitrate δ^{15}N decreases from ~5.3‰ at ~1,200-m depth in the water column to ~2.5‰ at ~200 m (ref. 14). Thus, the FB-δ^{15}N record indicates proportionally large variations in N fixation[15]. The record of bulk sediment δ^{15}N at Site 999 shows a number of minor changes that coincide with changes in FB-δ^{15}N, but they are so weak as to be unnoticeable without the benefit of the larger FB-δ^{15}N changes (Supplementary Fig. 2). The apparent muting of the variations suggests an allochthonous N input to the bulk sediment that does not respond to open-ocean changes[17].

The FB-δ^{15}N variation is highly correlated with Earth's 19–23-kyr orbital precession cycle (Fig. 2a and Supplementary Figs 6, 8 and 10). FB-δ^{15}N minima consistently lag *G. ruber* calcite δ^{18}O minima by 4–7 kyr (Fig. 2c and Supplementary Figs 6 and 9), and they thus lag peak Northern Hemisphere summer insolation at the precession frequency band by 10–12 kyr (ref. 19). That is, minima in Caribbean FB-δ^{15}N (maxima in N fixation) occur during aphelion in the northern summer (precession maxima; Fig. 2a), which is the opposite of the precession phase that encourages Northern Hemisphere summer warming and deglaciation. Despite this precessional phasing, FB-δ^{15}N is higher (N fixation is reduced) during the major glacial periods (MIS 6 and MISs 4–2) relative to the interglacials (MISs 5 and 1). This implies that there are non-precessional step changes in FB-δ^{15}N at each of the major deglaciations (the transitions between MISs 6 and 5 and between MISs 2 and 1) and at the intensification of glaciation at the transition between MISs 5 and 4.

Temperature is recognized as a control on the distribution of N fixation, both in the ocean and on land[4]. Thus, ice-age cooling might be expected to reduce N fixation, consistent with the basic LGM–Holocene change in N fixation. However, the full record indicates that N fixation does not track Caribbean sea surface temperature (SST) as reconstructed from the Mg/Ca ratio of *G. ruber* at Site 999[20] (Fig. 2d and Supplementary Fig. 9c). SST rose early in the penultimate deglaciation (the transition between MISs 6 and 5) and had already begun to decline before N fixation reached the first of its three maxima in MIS 5, with the subsequent maxima in N fixation occurring against a relatively stable baseline of low SST (Fig. 2d and Supplementary Fig. 7a, b). Hence, SST change does not seem to explain the history of N fixation reconstructed from FB-δ^{15}N.

Iron is a critical nutrient for N fixers, and iron-bearing dust has been suggested to cause ice-age enhancements in N fixation[2,3]. In our comparison of the N fixation record at Site 999 with records of dust input, we include a new reconstruction of dust transport from ODP Site 658, a coring site off the coast of North Africa, which is the dominant source of dust to the Caribbean (Fig. 1). The Zr/Al ratio increases with high-energy aeolian transport (Supplementary Fig. 3). Focusing first on the large-scale glacial–interglacial changes, the FB-δ^{15}N record at Site 999 indicates that N fixation was, if anything, inversely correlated with Zr/Al at Site 658; reconstructed dust flux at Site 659[21], which is farther offshore; terrigenous material abundance at equatorial Atlantic Site 663[22]; and iron accumulation rate at Site 999[23] (Fig. 2e and Supplementary Fig. 4). The correlation of N fixation with dust over the precession cycle is more difficult to assess. North African aridity has

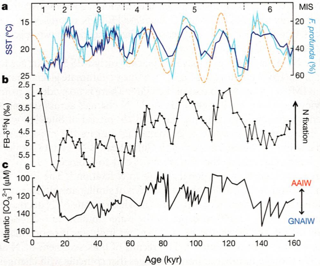

Figure 3 | Comparison of FB-δ^{15}N with changes in precession-paced equatorial Atlantic upwelling and glacial–interglacial Atlantic intermediate water source. **a**, *Florisphaera profunda* abundance (light blue) and foraminifera-species-derived SST (dark blue) from equatorial Atlantic core RC24-7[7]. **b**, FB-δ^{15}N from ODP Site 999. **c**, Bottom water carbonate ion reconstruction from Caribbean Sea core VM28-122[31] (12° N, 79° W; 3,620 m). The orange dashed line in **a** is the precession parameter. Episodes of higher equatorial Atlantic upwelling led to higher N fixation in the North Atlantic, observed as lower FB-δ^{15}N. In **c**, the switches in North Atlantic intermediate water source between AAIW, the dominant source of excess P to the Atlantic[5], and GNAIW, which was low in [PO_4^{3-}] (refs 28,32) and presumably also in excess P, can be seen. Periods of inflow of AAIW stimulated N fixation.

been reported to increase during northern summer aphelion[24], when we observe higher N fixation. However, available records suggest, on balance, that dust input is out of phase with the precessional cycles in N fixation (Fig. 2 and Supplementary Information). All things considered, it does not seem that a dust-derived iron supply can explain the reconstructed N fixation changes.

North Atlantic N fixation might be expected to change so as to balance denitrification changes[13]. Bulk sediment δ^{15}N records near the major suboxic zones indicate a lower rate of water column denitrification during cold periods[11], in the same sense as the N fixation decrease that we reconstruct for the LGM, the glacial of MIS 4 and the glacial maximum of MIS 6 (Fig. 2f, g and Supplementary Fig. 5). However, beyond this coarsest scale, there are major differences. For example, in contrast to the sediment δ^{15}N records from the denitrification zones, FB-δ^{15}N at Site 999 during early MIS 3 is similar to that during the LGM, with these local δ^{15}N minima separated by the highest sustained δ^{15}N of the record at ~35 kyr ago in late MIS 3 (Fig. 2a). In addition, tropical Pacific bulk sediment δ^{15}N maxima (water column denitrification maxima) seem to be associated with minima in Earth's orbital precession cycle (Fig. 2f), when perihelion coincides with northern summer. The FB-δ^{15}N record indicates that these times represent minima, not maxima, in Atlantic N fixation. Although a global ocean reconstruction of denitrification does not yet exist, the available records do not provide clear signs that changes in water column denitrification are responsible for the timing of changes in North Atlantic N fixation over the most recent glacial cycle.

The importance of precession in Caribbean FB-δ^{15}N suggests a connection to low-latitude climate. Over recent glacial cycles, equatorial Atlantic upwelling varied with a precession frequency[7]. In the eastern equatorial Atlantic, climatic precession maxima (aphelion in northern summer) coincide with minima in reconstructed SST (Fig. 3a). In addition, these times are characterized by minima in the fraction of the nanofossils represented by the nutricline dwelling form *Florisphaera profunda* (Fig. 3a), reflecting a shoaling of the nutricline[7]. These observations indicate that precession maxima yielded stronger equatorial Atlantic upwelling[7].

Precession-paced change in the equatorial Atlantic upwelling, coupled with a tendency of N fixation to occur in N-depleted, phosphorus-bearing water[6], explains the precession signal in Caribbean FB-δ^{15}N. Compared with the thermocline and surface layer of the western North Atlantic, subsurface water upwelled along the Equator has higher 'excess P'[5,6] (defined as the phosphate concentration minus 1/16th of the nitrate concentration; Fig. 1). In waters with positive excess P, phytoplankton tends to deplete the water of nitrate before the complete consumption of phosphate. The residual phosphate in this nitrate-free water should encourage the growth of N-fixing phytoplankton[13]. In line with this expectation, data-assimilating model calculations suggest that the transport into the tropical North Atlantic of equatorial water bearing excess P leads to a band of high N fixation in this region[6,25]. Moreover, N fixation seems to respond in the equatorial Atlantic upwelling region itself, before lateral transport of surface waters[26]. The excess P of the waters upwelled along the Equator originates in sub-Antarctic mode water (SAMW) and Antarctic intermediate water[5] (AAIW). With climatic precession maxima, increased equatorial upwelling of excess-P-bearing water coupled with northwestward transport of equatorial surface waters into the western tropical North Atlantic by the north Brazil current would increase the supply of excess P to the latter region, intensifying N fixation there (Fig. 3). Although the Amazon outflow may also contribute to the excess P in the north Brazil current (Fig. 1), its response to precession[27] seems to be inconsistent with our reconstructed N fixation changes.

The baseline shifts in FB-δ^{15}N at the respective transitions between MISs 6 and 5, 5 and 4, and 2 and 1 parallel well-known changes in the depth of North Atlantic ventilation (Fig. 3c): glacial North Atlantic intermediate water (GNAIW) formed during glacial stages 6 and 4–2, rather than the North Atlantic Deep Water (NADW) of interglacial stages[8]. GNAIW formation reduced the nutrient concentration at the base of the Atlantic thermocline[28] by preventing the influx of AAIW and SAMW or by diluting their impact, or both (Fig. 4). The low nutrient content of GNAIW implies that it formed from nutrient-depleted, low-latitude North Atlantic surface water, which today lacks excess P (ref. 5). Thus, the switch from NADW to GNAIW lowered the excess P of the subsurface waters to be upwelled (Fig. 4). A plausible alternative for the step changes in FB-δ^{15}N is that Atlantic N fixation was responding to sea-level-driven reductions in sedimentary denitrification during glacial stages 6 and 4–2. However, a shelf-associated decrease in sedimentary denitrification would have lowered excess P in the low-latitude upper ocean within decades to centuries of the sea-level decline at the onset of MIS 4. The FB-δ^{15}N increase in glacial stage 4 occurred ~7 kyr after the sea-level decrease (Fig. 2a, b and Supplementary Fig. 9a), inconsistent with a response to regional sedimentary denitrification but consistent with the timing of the switch to GNAIW[8].

Although variations in Atlantic N fixation do not seem to have paralleled global water column denitrification over the past 160 kyr, this does not require an imbalance between N fixation and denitrification on a global ocean basis; indeed, a sensitivity to phosphorus would encourage a global balance[13]. At the same time, this study recalibrates our expectations regarding the regional drivers of biogeochemical change in the low-nutrient tropical and subtropical ocean. Water column denitrification is broadly recognized as being sensitive to the physical circulation, given that the major ocean suboxic regions are located in the tropical 'shadow zones', the poorly ventilated subsurface volumes east of the subtropical gyres. Our results indicate that ocean circulation, through its control of nutrient transport, can also drive changes in regional rates of N fixation[6].

Although iron limitation of phytoplankton is fundamental to ocean biogeochemistry[2,3,5], the data reported here suggest that, in the North Atlantic, the rate of N fixation over the past 160 kyr has not been tightly constrained by iron supply. The entire ocean N and P reservoirs must flow through the North Atlantic on the timescale of ocean ventilation (~1 kyr), which is probably shorter than the residence time of the ocean's fixed N reservoir[1]. Thus, if N fixation in the rest of the ocean is significantly limited

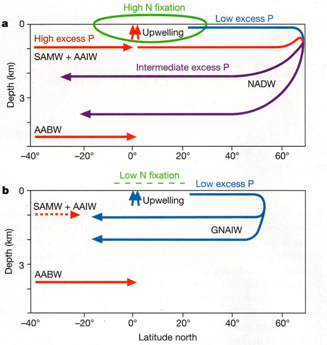

Figure 4 | Effect of changes in glacial–interglacial circulation on N fixation in the Atlantic. **a**, During the Holocene and MIS 5, SAMW and AAIW import high excess P (red) to the low latitudes, where its upwelling stimulates N fixation, which removes the excess P (conversion from red to blue). North Atlantic surface water, SAMW and AAIW are incorporated into newly formed NADW, which is thus intermediate in excess P (purple; ref. 5). **b**, In glacial stages MIS 6 and MISs 4–2 (where MIS 2 is the LGM), the low excess P of southward-flowing GNAIW would have diluted or replaced the high excess P of SAMW and AAIW, reducing the concentration of excess P available to be upwelled, and thus lowering N fixation. The precession-paced changes in equatorial Atlantic upwelling are not indicated in this diagram.

by iron, then the North Atlantic may have an important role in maintaining the relationship between the global ocean N and P reservoirs.

METHODS SUMMARY

FB-δ^{15}N was analysed by oxidizing organic N to nitrate and then using a bacterial method to convert the nitrate to nitrous oxide for isotope ratio mass spectrometry[15]. The individual species were picked manually, to obtain 3–7 mg of foraminifera per sample. The picked sample was crushed and underwent reductive and then oxidative cleaning to remove all N not bound within (and thus physically protected by) the calcite walls of the foraminifera tests. Dissolution of the tests with HCl released the test-bound organic N, which was then oxidized to nitrate. Sediment Zr/Al was measured with an Avaatech XRF Core Scanner at MARUM, University of Bremen. Data are of X-ray fluorescence count ratios and were obtained at a resolution of 1 cm over an area of 1.2 cm^2 directly at the split-core surface of the archive half.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions M.S., D.M.S. and G.H.H. designed the study. M.S. performed the FB-δ^{15}N analysis and wrote the first version of the manuscript with D.M.S.; A.N.M. generated the Zr/Al record at ODP Site 658. A.M.-G. performed the statistical analysis of the data with M.S.; H.R. was involved in the laboratory and data analysis. All authors contributed to the interpretation of the data and provided significant input to the final manuscript.

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LETTER

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Retardation of arsenic transport through a Pleistocene aquifer

Alexander van Geen[1], Benjamín C. Bostick[1], Pham Thi Kim Trang[2], Vi Mai Lan[2], Nguyen-Ngoc Mai[2], Phu Dao Manh[2], Pham Hung Viet[2], Kathleen Radloff[1]†, Zahid Aziz[1]†, Jacob L. Mey[1,3], Mason O. Stahl[4], Charles F. Harvey[4], Peter Oates[5], Beth Weinman[6]†, Caroline Stengel[7], Felix Frei[7], Rolf Kipfer[7,8] & Michael Berg[7]

Groundwater drawn daily from shallow alluvial sands by millions of wells over large areas of south and southeast Asia exposes an estimated population of over a hundred million people to toxic levels of arsenic[1]. Holocene aquifers are the source of widespread arsenic poisoning across the region[2,3]. In contrast, Pleistocene sands deposited in this region more than 12,000 years ago mostly do not host groundwater with high levels of arsenic. Pleistocene aquifers are increasingly used as a safe source of drinking water[4] and it is therefore important to understand under what conditions low levels of arsenic can be maintained. Here we reconstruct the initial phase of contamination of a Pleistocene aquifer near Hanoi, Vietnam. We demonstrate that changes in groundwater flow conditions and the redox state of the aquifer sands induced by groundwater pumping caused the lateral intrusion of arsenic contamination more than 120 metres from a Holocene aquifer into a previously uncontaminated Pleistocene aquifer. We also find that arsenic adsorbs onto the aquifer sands and that there is a 16–20-fold retardation in the extent of the contamination relative to the reconstructed lateral movement of groundwater over the same period. Our findings suggest that arsenic contamination of Pleistocene aquifers in south and southeast Asia as a consequence of increasing levels of groundwater pumping may have been delayed by the retardation of arsenic transport.

This study reconstructs the initial phase of contamination of an aquifer containing low levels of arsenic (low-As) in the village of Van Phuc, located 10 km southeast of Hanoi on the banks of the Red River. A key feature of the site is the juxtaposition of a high-As aquifer upstream of a low-As aquifer in an area where pumping for the city of Hanoi has dominated lateral groundwater flow for the past several decades (Fig. 1a). Many residents of the village of Van Phuc still draw water from their 30–50-m-deep private wells. In the western portion of the village, the wells typically contain less than 10 μg of As per litre of water and therefore meet the World Health Organization guideline for As in drinking water, whereas As in the groundwater from most wells in eastern Van Phuc exceeds this guideline by a factor of 10–50 (ref. 5).

Drilling and sediment dating in the area has shown that low-As groundwater is drawn from orange-coloured sands deposited over 12,000 years ago, whereas high-As groundwater is typically in contact with grey sands deposited less than 5,000 years ago[6,7]. We examined to what extent the boundary between the low-As and high-As aquifers of Van Phuc has shifted in response to groundwater withdrawals in Hanoi. This large-scale perturbation spanning several decades has implications for low-As aquifers throughout Asia that are vulnerable to contamination owing to accelerated groundwater flow.

The collection of sediment cores and the installation of monitoring wells was concentrated along a transect trending southeast to northwest that extends over a distance of 2.2 km from the bank of the Red River (Fig. 1b). Groundwater heads, and therefore the groundwater velocity field, within Van Phuc respond rapidly to the daily and seasonal cycles in the water level of the river (Supplementary Information). Before large-scale groundwater withdrawals, rainfall was sufficient to maintain groundwater discharge to the river, as is still observed elsewhere along the Red River[8]. In Van Phuc, however, the groundwater level was on average 40 cm below that of the water level of the Red River in 2010–11 and the hydraulic gradient nearly always indicated flow from the river into the aquifer. The reversal of the natural head gradient is caused by the large depression in groundwater level centred 10 km to the northwest that induces groundwater flow along the Van Phuc transect from the river towards Hanoi (Fig. 1a). This perturbation of groundwater flow is caused by massive pumping for the municipal water supply of Hanoi[9–11], which nearly doubled from 0.55 million to 0.90 million cubic metres per day between 2000 and 2010 owing to the rapid expansion of the city (Supplementary Fig. 1).

A change in the colour of a clay layer capping sandy sediment along the transect defines a geological boundary between the two portions of the Van Phuc aquifer. Up to a distance of 1.7 km from the river bank, the clay capping the aquifer is uniformly grey with the exception of a thin brown interval at the very surface (Fig. 2b). In contrast, a readily identifiable sequence of highly oxidized bright yellow, red and white clays was encountered between 12 m and 17 m depth at all drill sites along the transect beyond a distance of 1.7 km from the river bank. This oxidized clay layer is probably a palaeosol dating to the last sea-level low-stand about 20,000 years ago[7,12].

The colour of aquifer sands below the upper clay layer also changes markedly along the Van Phuc transect. Sand colour in fluvio-deltaic deposits is controlled primarily by the extent to which Fe(III) has been reduced to Fe(II) by the decomposition of organic carbon[13]. Up to a distance of 1.6 km from the river bank, sandy drill cuttings within the 20–40 m depth range are uniformly grey. The predominance of orange sands beyond 1.6 km indicates oxidation during the previous sea-level low-stand. After the sea level rose back to its current level, the nature of the remaining organic carbon precluded a new cycle of Fe(III) reduction[14].

Independently of sediment colour, the calcium (Ca) content of sand cuttings collected while drilling along the Van Phuc transect confirms that a geological boundary extends to the underlying aquifer sands. Within the southeastern portion of the aquifer that is not capped by the presumed palaeosol, X-ray fluorescence measurements indicate Ca concentrations of over 2,000 mg Ca per kg of sand in cuttings to a depth of 30 m (Fig. 2a). The groundwater in this portion of the aquifer is supersaturated with respect to calcite and dolomite[6], suggesting that authigenic precipitation is the source of Ca in the grey drill cuttings, as previously proposed elsewhere[12] (Supplementary Fig. 2). At a distance of 1.7 km from the river and further to the northwest, instead, the Ca

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[1]Lamont-Doherty Earth Observatory (LDEO), Columbia University, Palisades, New York 10964, USA. [2]Research Centre for Environmental Technology and Sustainable Development (CETASD), Hanoi University of Science, Vietnam National University, Hanoi, Vietnam. [3]Department of Physical Sciences, Kingsborough Community College, Brooklyn, New York 11235, USA. [4]Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA. [5]Anchor QEA, Montvale, New Jersey 07645, USA. [6]Earth and Environmental Sciences, Vanderbilt University, Nashville, Tennessee 37235, USA. [7]Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland. [8]Institute of Geochemistry and Petrology, Swiss Federal Institute of Technology, Zurich ETHZ 8092, Switzerland.
†Present addresses: Gradient, Cambridge, Massachusetts 02138, USA (K.R.); Sadat Associates, Trenton, New Jersey 08610, USA (Z.A.); Earth and Environmental Sciences, California State University, Fresno, California 93740, USA (B.W.).

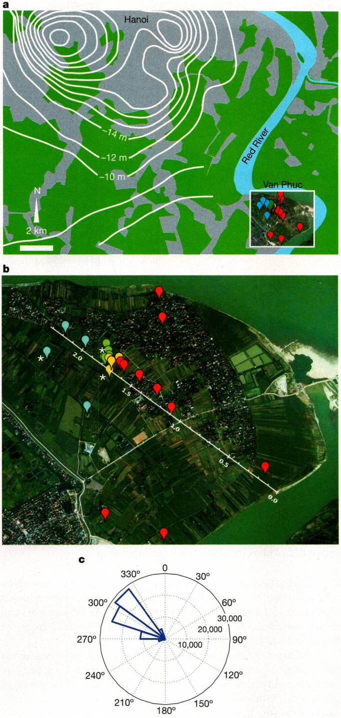

Figure 1 | Map of the Hanoi area extending south to the study site. **a**, Location of the village of Van Phuc in relation to the cone of depression formed by groundwater pumping for the municipal water supply of Hanoi (white contours, adapted from ref. 10). Urbanized areas are shown in grey; largely open fields are shown in green. **b**, Enlarged view of Van Phuc (box shows location in **a**) from Google Earth showing the location of the transect along which groundwater and sediment were collected, with tickmark labels indicating distance from the Red River bank in kilometres. Symbol colour distinguishes the uniformly grey Holocene aquifer (red), the Pleistocene aquifer contaminated with As (yellow), the Pleistocene aquifer where the groundwater conductivity and dissolved inorganic carbon concentrations are high but As concentrations are not (green), and the Pleistocene aquifer without indication of contamination (blue), all within the 25–30-m depth interval. Three white asterisks identify the wells that were used to determine flow direction. Image copyright 2012 Digital Globe Google Earth. **c**, Rose diagram frequency plot of the head gradient direction based on data collected at 5-min intervals (numbers indicate the number of observations) from these three wells from September 2010 to June 2011.

content of orange sand cuttings systematically remains less than 100 mg Ca per kg and the groundwater is undersaturated with respect to calcite and dolomite. Unlike surficial shallow grey clays, the Ca content of the presumed palaeosol is also very low (<100 mg Ca per kg) and consistent with extensive weathering.

The redox state of the aquifer has a major impact on the composition of groundwater in Van Phuc, as reported elsewhere in Vietnam[15] and across south and east Asia more generally[3]. High but harmless Fe(II) concentrations in groundwater (10–20 mg per litre) associated with grey reducing sediments are apparent to residents of eastern Van Phuc as an orange Fe(III) precipitate that forms in their water upon exposure to air (Supplementary Fig. 3). In contrast, the high and toxic concentrations of As in groundwater at 20–30 m depth within the same portion of the transect, ranging from 200 μg per litre near the river to levels as high as 600 μg per litre at 1.2–1.6 km from the river bank, are invisible (Fig. 2c). The groundwater in contact with Pleistocene sands in northwestern Van Phuc is also anaerobic but contains less than 0.5 mg Fe(II) per litre and less than 10 μg As per litre and shows little indication of organic carbon mineralization compared to the Holocene aquifer (Supplementary Fig. 4).

The Pleistocene portion of the Van Phuc aquifer adjacent to the Holocene sediment is not uniformly orange or low in As. Of particular interest is a layer of grey sand at 25–30 m depth extending to the northwest at a distance of 1.7–1.8 km from the river bank (Fig. 2b). The intercalation of grey sand between orange sands above and below, combined with the low Ca content of sand cuttings within this layer, indicate that it was deposited during the Pleistocene and therefore until recently oxidized and orange in colour. Within the portion of the Pleistocene aquifer that became grey and is closest to the geological boundary, groundwater As concentrations are therefore presumed to have been originally very low (<5 μg per litre). Actual As concentrations of 100–500 μg per litre, as high as in the adjacent Holocene aquifer, indicate contamination extending over a distance of about 120 m into the Pleistocene aquifer (Fig. 3a).

A subset of the transect wells was sampled in 2006 and analysed for tritium (^{3}H) as well as noble gases in order to measure groundwater ages and determine the rate of As intrusion into the Pleistocene aquifer. Atmospheric nuclear weapons testing in the 1950s and 1960s is the main source of ^{3}H that entered the hydrological cycle[16]. The distribution of ^{3}H indicates that only groundwater in the southeastern high-As portion of the aquifer contains a plume of recharge dating from the 1950s and later. Concentrations of ^{3}He, the stable decay product of ^{3}H, were used to calculate groundwater ages for eight wells in the 24–42-m depth range with detectable levels of ^{3}H. In 2006, the oldest water dated by the ^{3}H–^{3}He method (Supplementary Fig. 5) was sampled at a distance of 1.6 km from the river, which is the most northwestern location along the transect where the aquifer is uniformly grey (Fig. 2b, d). Younger ages of 15 years and 17 years were measured closer to the river at 1.3 km and 1.5 km, respectively. Concentrations of ^{3}H, groundwater ^{3}H–^{3}He ages, and hydraulic head gradients consistently indicate that the Holocene aquifer has been recharged by the river from the southeast within the past few decades.

Drilling and geophysical data indicate that the main groundwater recharge area extends from the centre of the Red River to the inland area where a surficial clay layer thickens markedly, that is, from 100 m southeast to 300 m northwest of the river bank (Supplementary Fig. 6). The relationship between groundwater ages and travel distance from the recharge area implies accelerating flow drawn by increased Hanoi pumping (Supplementary Fig. 7). A simple transient flow model for the Van Phuc aquifer yields average advection rates of 38 m yr^{-1} and 48 m yr^{-1} towards Hanoi since 1951 and 1971, respectively (Supplementary Discussion). According to these two pumping scenarios,

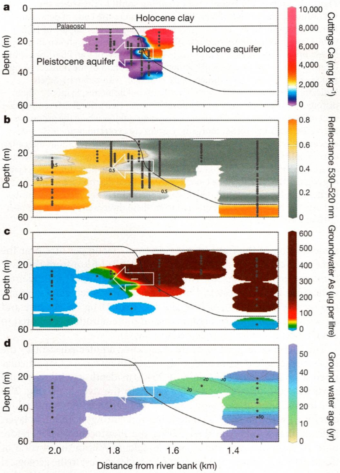

Figure 2 | Contoured sections of sediment and water properties based on data collected between 1.3 km and 2.0 km from the Red River bank. The location and number of samples indicated as black dots varies by type of measurement. **a**, Concentration of Ca in sand cuttings measured by X-ray fluorescence. Also shown are the boundaries separating the two main aquifers and the palaeosol overlying the Pleistocene aquifer. '2000' labels the contour for 2,000 mg Ca per kg. **b**, Difference in diffuse spectral reflectance between 530 nm and 520 nm, indicative of the colour of freshly collected drill cuttings[13]. The contour labels correspond to the percentage difference in reflectance shown by the colour scale. **c**, Concentrations of As in groundwater collected in 2006 with the needle sampler and in 2011 by monitoring wells along the transect. '10' labels the contour for the WHO guideline, 10 µg As per litre. **d**, Groundwater ages relative to recharge determined by ^{3}H–^{3}He dating of groundwater samples collected from a subset of the monitoring wells in 2006. The portion of the Pleistocene aquifer that became reduced and where As concentrations presumably increased over time is located within the large white arrow pointing in the direction of flow. The plot was drawn with Ocean Data View (http://odv.awi.de/).

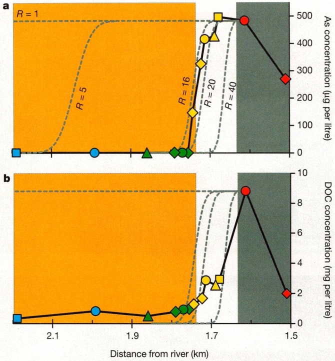

Figure 3 | Distribution of arsenic and dissolved organic carbon in groundwater within the 25–30-m depth interval along the Van Phuc transect. Symbols are coloured according to the classification in Fig. 1. Grey and orange shading indicates the extent of the grey Holocene aquifer and the portion of the Pleistocene aquifer that is still orange, respectively. The intermediate area without shading indicates the portion of the Pleistocene aquifer that became grey. Shown as dotted lines are predicted As concentrations bracketing the observations with retardation factors *R* of 16 and 20 and an average advection velocity of 43 m yr^{-1} over the 50 years preceding the 2011 sampling (Supplementary Discussion). **a**, Also shown are predicted concentrations for As assuming retardation factors of 1, 5 and 40 and the same average rate of advection. **b**, For visual reference, predicted dissolved organic carbon concentrations are shown as dotted lines according to the same advection velocity and retardation factors of 16, 20 and 40, assuming there was no detectable dissolved organic carbon in the Pleistocene aquifer before the perturbation.

groundwater originating from the Holocene portion of the aquifer was transported 2,000–2,300 m into the Pleistocene sands by 2011, when the transect was sampled for analysis of As and other groundwater constituents.

The sharp decline in As concentrations between 1.60 km and 1.75 km from the river bank indicates that migration of the As front across the geological boundary was retarded by a factor of 16 to 20 relative to the movement of the groundwater (Fig. 3a). Without retardation, attributable to As adsorption onto aquifer sands, the entire Pleistocene aquifer of Van Phuc would already be contaminated. The retardation is derived from several decades of perturbation and is at the low end of previous estimates by other methods, typically measured within days to weeks[17–22], and therefore predicts greater As mobility than most previous studies. The retardation measured in Van Phuc integrates the effect of competing ions typically present at higher concentrations in the Holocene aquifer (Supplementary Fig. 4) as well as the impact of Fe oxyhydroxide reduction. However, the extent to which contamination was caused by either As transport from the adjacent Holocene aquifer or reductive dissolution of Fe(III) oxyhydroxides and *in situ* As release to groundwater cannot be determined from the available data (Supplementary Fig. 8).

The sharp drop in dissolved organic carbon concentrations across the geological boundary from 9 mg per litre to about 1 mg per litre indicates rapid organic carbon mineralization coupled to the reduction of Fe(III) oxyhydroxides and explains the formation of a plume of grey sands within the Pleistocene aquifer (Fig. 3b). On the basis of a stoichiometric Fe/C ratio of 4 (ref. 15), the dissolved organic carbon supplied by flushing the aquifer 30 times with groundwater from the Holocene aquifer would be required to turn Pleistocene sands from orange to grey by reducing half of their 0.1% reactive Fe(III) oxyhydroxide content[23], assuming a porosity of 0.25. Given that groundwater was advected over a distance of 2,000–2,300 m across the geological boundary over the past 40–60 years, we would predict that the plume of grey sands extends 65–75 m into the Pleistocene aquifer. This is somewhat less than is observed (Fig. 3), possibly due to additional reduction by H_2 advected from the Holocene portion of the aquifer[14]. The Van Phuc observations indicate that dissolved organic carbon advected from a Holocene aquifer can be at least as important for the release of As to groundwater as autochthonous organic carbon[12,24–27].

Contamination of Pleistocene aquifers has previously been invoked in the Red River and the Bengal basins[11,12,28], but without the benefit of a well-defined hydrogeological context. The Pleistocene aquifer of Van Phuc was contaminated under the conducive circumstances of accelerated lateral flow. Although downward groundwater flow and therefore penetration of As will typically be slower, the Van Phuc findings confirm that the vulnerability of Pleistocene aquifers will depend on the local spatial density of incised palaeo-channels that were subsequently filled with Holocene sediments[12]. Owing to retardation, concentrations of As in a Pleistocene aquifer will not increase suddenly but over timescales of decades even in the close vicinity of a Holocene aquifer. This is consistent with the gradual increase in groundwater As concentrations documented by the few extended time series available from such a vulnerable setting[29]. However, concentrations of As could rise more rapidly where flow accelerates beyond the rate documented in Van Phuc, closer to Hanoi for instance.

METHODS SUMMARY

A total of 41 wells were installed in Van Phuc in 2006–11. The water levels of the river and in the wells were recorded from September 2010 to June 2011 using pressure transducers and adjusted to the same elevation datum after barometric corrections. The magnitude and direction of the head gradient within the 25–30-m depth interval was calculated from water level measurements in three wells (Fig. 1b). In 2006, a subset of the wells was sampled for noble gas and tritium (^{3}H) analysis at a high flow rate using a submersible pump to avoid degassing. The samples were analysed by mass spectrometry in the Noble Gas Laboratory at ETH Zurich. ^{3}H concentrations were determined by the ^{3}He ingrowth method[30]. Groundwater As, Fe and Mn concentrations measured by high-resolution inductively coupled plasma mass spectrometry at LDEO represent the average for acidified samples collected in April and May 2012. Further details are provided in the Supplementary Information.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Non-chondritic sulphur isotope composition of the terrestrial mantle

J. Labidi[1], P. Cartigny[1] & M. Moreira[2]

Core–mantle differentiation is the largest event experienced by a growing planet during its early history. Terrestrial core segregation imprinted the residual mantle composition by scavenging siderophile (iron-loving) elements such as tungsten, cobalt and sulphur. Cosmochemical constraints suggest that about 97% of Earth's sulphur should at present reside in the core[1], which implies that the residual silicate mantle should exhibit fractionated $^{34}S/^{32}S$ ratios according to the relevant metal–silicate partition coefficients[2], together with fractionated siderophile element abundances. However, Earth's mantle has long been thought to be both homogeneous and chondritic for $^{34}S/^{32}S$, similar to Canyon Diablo troilite[3–6], as it is for most siderophile elements. This belief was consistent with a mantle sulphur budget dominated by late-accreted chondritic components. Here we show that the mantle, as sampled by mid-ocean ridge basalts from the south Atlantic ridge, displays heterogeneous $^{34}S/^{32}S$ ratios, directly correlated to the strontium and neodymium isotope ratios $^{87}Sr/^{86}Sr$ and $^{143}Nd/^{144}Nd$. These isotope trends are compatible with binary mixing between a low-$^{34}S/^{32}S$ ambient mantle and a high-$^{34}S/^{32}S$ recycled component that we infer to be subducted sediments. The depleted end-member is characterized by a significantly negative $\delta^{34}S$ of -1.28 ± 0.33‰ that cannot reach a chondritic value even when surface sulphur (from continents, altered oceanic crust, sediments and oceans) is added. Such a non-chondritic $^{34}S/^{32}S$ ratio for the silicate Earth could be accounted for by a core–mantle differentiation record in which the core has a $^{34}S/^{32}S$ ratio slightly higher than that of chondrites ($\delta^{34}S = +0.07$‰). Despite evidence for late-veneer addition of siderophile elements (and therefore sulphur) after core formation, our results imply that the mantle sulphur budget retains fingerprints of core–mantle differentiation.

Earlier reports of mid-ocean ridge basalts (MORBs) $\delta^{34}S$ (where $\delta^{34}S = [(^{34}S/^{32}S)_{sample}/(^{34}S/^{32}S)_{CDT} - 1] \times 1{,}000$ and CDT is Canyon Diablo troilite) yielded values statistically indistinguishable from the reported chondrite average of 0.04 ± 0.31‰ (refs 3–6), consistent with a late-veneer origin for sulphur in the mantle. This view has been recently questioned in a study using improved sulphur-extraction techniques[7]. Worldwide MORBs exhibit exclusively negative $\delta^{34}S$ ranging down to -1.9‰, with an approximately 2‰ variability[7]. These observations raise questions as to whether the terrestrial mantle is chondritic in sulphur isotopes, but the mechanisms controlling MORB $^{34}S/^{32}S$ variability remain unclear.

To address these issues, we investigated the sulphur isotope composition of 23 glasses dredged on the South Atlantic ridge between 40° and 55° S. Previous radiogenic isotopes[8–10], noble gases[11,12], volatiles[13], major-element[14] and trace-element[15] measurements on these basalts illustrate interactions of the Shona–Discovery hotspots with the ridge. Several of the typical mantle end-members feed the mantle source of the two plumes, including HIMU ('high-μ' where $\mu = ^{238}U/^{204}Pb$), LOMU ('low-μ'), and enriched-mantle components. The samples analysed were chosen to reflect this geochemical variability and thus offer an opportunity to address $^{34}S/^{32}S$ variations with respect to these mantle heterogeneities.

In our samples, sulphur occurs only in its reduced form, as commonly found in MORBs[16–18], and the sulphur content, from 642 to 1,388 parts per million (p.p.m.), correlates with the FeO content (Supplementary Fig. 3A), illustrating magmatic sulphide saturation[16]. The eruption depth of our samples precludes any significant sulphur degassing[19], restricting the sulphur isotope discussion to primary magmatic considerations. $\delta^{34}S$ varies between -1.80‰ and $+1.05$‰, whereas both the quantifying mass-independent signatures $\Delta^{33}S$ and $\Delta^{36}S$ (where $\Delta^{33}S = \delta^{33}S - 1{,}000 \times [(\delta^{34}S/1{,}000 + 1)^{0.515} - 1]$ and $\Delta^{36}S = \delta^{36}S - 1{,}000 \times [(\delta^{34}S/1{,}000 + 1)^{1.889} - 1]$), are homogeneous and equal within uncertainty to those of Canyon Diablo troilite (Supplementary Table 1). Besides, $\delta^{34}S$ is remarkably correlated to source enrichment proxies such as $^{87}Sr/^{86}Sr$ or $^{143}Nd/^{144}Nd$ (Fig. 1). Seawater sulphate ($\delta^{34}S = +21$‰) assimilation by erupted basalts could result in an increase in $\delta^{34}S$; it would, however, scatter or erase any correlations between $\delta^{34}S$ and tracers that remain insensitive to seawater incorporation (such as $^{143}Nd/^{144}Nd$). It would also result in a relationship between $\delta^{34}S$ and proxies of seawater incorporation such as chlorine contents or Cl/K ratios, which is again not the case (Supplementary Fig. 4). Correlated $\delta^{34}S$ and radiogenic isotope enrichments thus reflect the variability of the MORB sources.

All the samples are saturated with immiscible sulphide[16], and the extremely low osmium contents in the most primitive MORBs probably indicates that they were already sulphide-saturated during melting[10]. Such a saturation mechanism prevents us from constraining the sulphur contents of the mantle source, and because the fractionation factor between dissolved sulphur and magmatic sulphide might be significant, it raises the question of whether basalts can preserve the $^{34}S/^{32}S$ ratios of their mantle source. Those basalts investigated have such distinct differentiation and melting histories[14,15] that the amount of sulphide left during both melting and differentiation is variable[10,16]. If the high-temperature sulphur isotope fractionation during magmatic sulphide exsolution were significant, it would have erased the observed $\delta^{34}S$–$^{87}Sr/^{86}Sr$–$^{143}Nd/^{144}Nd$ trends (Fig. 1). The lack of correlation between $\delta^{34}S$ and sulphide segregation proxies indicates a fractionation factor, $\alpha_{sulph\text{-}melt}$, of between 1.0000 and 0.9995 ± 0.0005, at most (Supplementary Information). The $\delta^{34}S$ of basalts hence provides direct information on mantle source composition, here displaying an average value of -0.80 ± 0.58‰ (1σ).

The most ^{34}S-depleted samples correspond to depleted MORB and ^{34}S-enriched samples are related to the Discovery plume, whereas the Shona and LOMU basalts are of intermediate composition. Interestingly, samples from Shona and Discovery display similar $^{3}He/^{4}He$ and $^{21}Ne/^{22}Ne$ (refs 11 and 12) but have different ^{34}S enrichments, suggesting that these isotopic systems are decoupled. Such a lack of correlation between noble gases and S isotopes suggests that the primitive mantle is sulphur-poor compared to the recycled components. Hence, characterization of the primordial sulphur cannot be directly achieved from the present data set.

Our results can be explored in two ways. First, the south Atlantic depleted mantle has an average $\delta^{34}S$ of -1.28 ± 0.33‰ (error is 1σ,

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[1]Laboratoire de Géochimie des Isotopes Stables, Institut de Physique du Globe de Paris, Sorbonne Paris Cité, Université Paris Diderot, UMR 7154 CNRS, 1 rue Jussieu, 75005 Paris, France. [2]Laboratoire de Géochimie et Cosmochimie, Institut de Physique du Globe de Paris, Sorbonne Paris Cité, Université Paris Diderot, UMR 7154 CNRS 1 rue Jussieu, 75005 Paris, France.

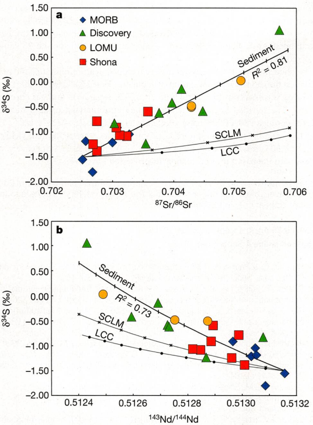

Figure 1 | δ^{34}S versus ^{87}Sr/^{86}Sr and ^{143}Nd/^{144}Nd. Strontium and neodymium isotope data are from ref. 8. Sulphur-isotope uncertainties are estimated on the basis of replicate analysis for all samples, and are all within symbol sizes. The samples define a binary mixing relationship between a depleted-mantle and an enriched-mantle endmember. Mixing trends with sub-continental lithospheric mantle (SCLM) and lower continental crust (LCC) are also shown, illustrating that mixing with sediments account better for the geochemical composition of our samples. **a**, δ^{34}S versus ^{87}Sr/^{86}Sr. **b**, δ^{34}S versus ^{143}Nd/^{144}Nd. δ^{34}S is defined in the main text; the CDT standard is used. *R*, correlation coefficient. Data used for the mixing calculation is as follows, from refs 22–27 and Supplementary ref. 37. For depleted mantle, $\delta^{34}S = -1.5‰$, $^{87}Sr/^{86}Sr = 0.7025$, $^{143}Nd/^{144}Nd = 0.513156$, Sr = 11.3 p.p.m., Nd = 1.12 p.p.m. and S = 200 p.p.m. For LCC, $\delta^{34}S = +3‰$, $^{87}Sr/^{86}Sr = 0.7080$, $^{143}Nd/^{144}Nd = 0.511600$, Sr = 348 p.p.m., Nd = 11 p.p.m. and S = 408 p.p.m. For SCLM, $\delta^{34}S = +3‰$, $^{87}Sr/^{86}Sr = 0.7100$, $^{143}Nd/^{144}Nd = 0.511663$, Sr = 49 p.p.m., Nd = 2.67 p.p.m. and S = 157 p.p.m. For the sediment, $\delta^{34}S = +10‰$, $^{87}Sr/^{86}Sr = 0.7203$, $^{143}Nd/^{144}Nd = 0.511170$, Sr = 327 p.p.m., Nd = 27 p.p.m. and S = 5,700 p.p.m. Markers on the fits are separated by 0.1%, 1.0% and 4.0% for sediment, LCC and SCLM, respectively.

n = 6), defining a mean value consistently distinct from chondrites. We note that an extrapolation of Sr–Nd–S isotope trends to most depleted compositions would lead to a depleted-mantle endmember as low as −1.80‰ (Fig. 1). Second, the nature of classical mantle endmembers can be addressed. Whereas it is commonly accepted that HIMU features reflect the occurrence of subducted oceanic crust in plume sources[20], the enriched-mantle and LOMU cases remain much debated. In the south Atlantic mantle, a significant contribution of continental material has been invoked to account for the LOMU endmember, including either delaminated subcontinental lithospheric mantle[8,9] or lower continental crust[10]. In S–Sr and S–Nd isotope spaces, however, the samples lie on a linear trend regardless of their locations or type of anomalies (Fig. 1), highlighting binary mixing between depleted mantle ($\delta^{34}S = -1.28‰$ or lower) and the enriched-mantle-type endmember ($\delta^{34}S = +1.05‰$ or higher).

Both LOMU basalts (having the lowest ^{206}Pb/^{204}Pb) and Shona samples (HIMU-type[8], having the highest ^{206}Pb/^{204}Pb) cannot be distinguished from the other samples in the observed trends (Fig. 1). This lack of correlation between δ^{34}S and ^{206}Pb/^{204}Pb (Supplementary Fig. 5), together with the preservation of the trends between δ^{34}S and Sr–Nd isotopes, argue in favour of relatively sulphur-poor HIMU and LOMU endmembers, overprinted by the contribution of a sulphur-rich enriched-mantle component. In this view, recycled oceanic crust carrying the radiogenic ^{206}Pb/^{204}Pb ratio is inferred to be relatively sulphur-depleted, consistent with its required high U/Pb. Sulphides are indeed the main Pb carrier in the oceanic crust[21] and any Pb loss along subduction should occur through a concomitant sulphur loss.

The mixing trends being linear, the enriched-mantle component must be enriched in sulphur in the same way as it is enriched in strontium and neodymium. This allows a reliable estimate of its S/Sr and S/Nd, broadly equal to that of the depleted mantle. For simplicity, only the S/Sr ratio is used in the following. Taking 200 ± 40 p.p.m. (ref. 22) for sulphur and 11.3 p.p.m. (ref. 15) for strontium in depleted mantle, we obtain an S/Sr value for enriched mantle of 17 ± 4. Both delaminated lithospheric mantle and lower continental crust have been suggested to account for enriched-mantle-type signals, especially in south Atlantic basalts[8–10]. These reservoirs are, however, relatively sulphur-poor compared to incompatible trace elements, yielding S/Sr ratios of 3.2 ± 2.0 (ref. 23) and 0.8 ± 0.3 (ref. 24), respectively, that are low compared to the required value of 17 ± 4 (see above). Any occurrence of such a component in the south Atlantic mantle would have led to highly curved mixing relationships in S–Sr isotope space, especially given the range of ^{87}Sr/^{86}Sr displayed by the samples (Fig. 1a).

Alternatively, sediment may be a good candidate: sediments are enriched in trace elements[25] and can bear significant amounts of sulphur. In particular, many sediments deposited one to two billion years ago during the Proterozoic eon formed under reduced conditions and usually contain more than 1% of sulphur pyrite[26]. A subducted sediment containing a realistic sulphur content of 5,700 ± 1,000 p.p.m. would satisfy the S/Sr of the enriched-mantle component. The fact that our estimate for recycled sediment falls within large uncertainties of non-subducted sediments suggests only moderate devolatilization, if any, during recycling (Supplementary Information). The best fits to our data are obtained for sediment having a δ^{34}S of +10 ± 3‰, consistent with sediments one to two billion years old with an average δ^{34}S of +5 ± 10‰ (ref. 27). This Proterozoic age for subducted sediments is also consistent with the observed lack of Δ^{33}S variability[28] best-fitted with a Δ^{33}S of +0.06 ± 0.03‰ (Supplementary Fig. 6).

If subducted sediments are major ^{34}S carriers to the deep mantle, the depleted mantle is shown here to be significantly ^{34}S-depleted with respect to chondrites. This low ^{34}S/^{32}S ratio has fundamental implications for our understanding of the origin and processes affecting moderately volatile elements in the terrestrial mantle. In the following, we use the −1.28 ± 0.33‰ depleted MORB average as the representative δ^{34}S of the depleted mantle, but further studies are needed to refine this value. Such a negative estimate allows us to discard a purely late-veneer origin for sulphur in the mantle, because this scenario requires a strictly chondritic ^{34}S/^{32}S ratio for depleted mantle. According to the kinetic theory of gases, partial evaporation of sulphur during accretion could have modified the ^{34}S/^{32}S ratio of the accreted Earth, leaving the residual terrestrial mantle enriched in ^{34}S relative to chondrites. This would be at odds with the observed depletion in ^{34}S. Finally, assuming that the depleted mantle represents 25%–80% of the whole mantle[29], the complementary surface reservoirs (continents, oceans and altered oceanic crust) would need to have an average δ^{34}S of between +8‰ and +25‰ to balance the non-chondritic δ^{34}S of the depleted mantle. Current estimates, however, yield average δ^{34}S values of −0.4 ± 3.0‰ (ref. 30) for the bulk surface reservoirs, a value that is far from being reconcilable with the above requirement, and emphasizing the non-chondritic character of the bulk silicate Earth.

In contrast to immiscible sulphide exsolution, sulphur dissolution into metal from silicate could fractionate the ^{34}S/^{32}S ratio owing to the distinct molecular environment of sulphur in a silicate versus a metallic liquid (and distinct vibrational partition functions). One likely possibility

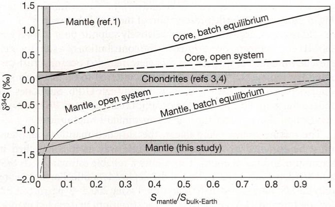

Figure 2 | Core–mantle sulphur partitioning model in batch equilibrium or open system. The $\delta^{34}S$ of the mantle is plotted against the sulphur content of the mantle S_{mantle} divided by the sulphur content of the bulk Earth $S_{\mathrm{bulk\text{-}Earth}}$. According to ref. 1, S_{mantle} represents less than 3% of $S_{\mathrm{bulk\text{-}Earth}}$. The sulphur isotope composition of chondrites (refs 3 and 4) and mantle (this study) values are both plotted, allowing a clear visualization of the sulphur isotope shift between these two reservoirs. Sulphur isotope and sulphur abundance observations coincide at an anchor point constraining the core–mantle fractionation. Under batch equilibrium, core $\delta^{34}S$ is +1.26‰ higher than the silicate value ($\alpha_{\mathrm{core-mantle}} = 1.00130$). In an open system model, the core is treated as a cumulative product and $\alpha_{\mathrm{core-mantle}}$ of 1.00035 is sufficient to explain the shift between mantle and chondrites.

involves the dissolution of sulphur in its metallic form (S^0) in the core, given that stable isotope fractionation theory predicts the more oxidized compound to be ^{34}S-enriched compared to sulphur dissolved as S^{2-} in the silicate mantle. The $\alpha_{\mathrm{core-mantle}}$ can be inferred from a chondritic bulk Earth (that is, mantle and core) by combining the distribution of sulphur in the bulk Earth (ref. 1) and the re-evaluated $^{34}S/^{32}S$ of the mantle (this study), leading to a $\alpha_{\mathrm{core-mantle}}$ of 1.00130 for a batch-equilibrium or 1.00035 for an open-system sulphur incorporation into the core (Fig. 2). In all cases, the core $\delta^{34}S$ is expected to be +0.07‰, almost indistinguishable from that of chondrites and iron meteorites, because this reservoir would contain most of the terrestrial sulphur[1].

Only Shahar *et al.*[2] experimentally addressed the $^{34}S/^{32}S$ fractionation between metal and silicate at high temperature, and estimated a $\alpha_{\mathrm{core-mantle}}$ of 1.00220 ± 0.0014‰ at 1,850 °C (ref. 2). For 97% of the sulphur occurring in the core[1], such an estimate should lead to a mantle $\delta^{34}S$ of −2.10 ± 1.40‰, under batch-equilibrium fractionation. This value, despite a significant overlap, seems slightly lower than our observation. Because stable isotope fractionations scale with temperature T as $1/T^2$, such distinct estimates could reflect a core segregation occurring at a temperature higher than 1,850 °C: the average temperature for core–mantle segregation remains highly debated, with values up to 3,300 °C (ref. 31). Alternatively, such a distinction may require the involvement of 'hybrid models', as suggested for highly siderophile elements[31], in which a fraction of the mantle sulphur is a fractionated leftover from core–mantle equilibrium, whereas the other fraction would have been delivered during the late-accretionary stage. For a $\alpha_{\mathrm{core-mantle}}$ of 1.00220 (ref. 2), the proportion of late-accreted sulphur (that is, with chondritic $^{34}S/^{32}S$) in the mantle would reach approximately 40% of mantle sulphur, after batch-equilibrium core segregation.

At that stage, the most obvious implication of such a core–mantle differentiation record is that sulphur and elements of comparable volatilities (such as zinc, fluorine and lead) must have been present to some extent before the late-accretion event. This is a robust and independent constraint that will help to build more consistent models of timing for the origin of moderately volatile elements in Earth's mantle.

METHODS SUMMARY

Samples were analysed following the protocol described in ref. 7. Glassy rims of basalts were handpicked under a binocular microscope and if needed, cleaned through a sonication in 99.9% ethanol. For each sample, about 300 mg was crushed to a fine powder (<100 μm) and dissolved in a 29 N HF + 2.1 M $CrCl_2$ solution. Under these conditions, sulphur is released as H_2S and subsequently trapped as precipitated Ag_2S. Sulphur extraction yields are 101 ± 4% for the 23 samples analysed in this study.

Weighed aliquots of silver sulphide were wrapped in aluminium foil and placed in Ni-reaction bombs for fluorination with purified F_2 at 250 °C overnight. The SF_6 produced was then purified using both cryogenic separation and gas chromatography. The purified SF_6 was then quantified and analysed using a dual-inlet ThermoFinnigan MAT 253 mass spectrometer where $m/z = 127^+, 128^+, 129^+$ and 131^+ ion beams are monitored. All the samples were replicated, yielding a ±0.1, ±0.005 and ±0.1‰ uncertainty for $\delta^{34}S$, $\Delta^{33}S$ and $\Delta^{36}S$, respectively, consistent with the basalt internal standards processed in ref. 7.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions J.L. performed sulphur isotope measurements. J.L. and M.M. performed sample preparation. P.C. conceived the project. J.L. took the lead in writing the paper, with substantial contributions from P.C. and M.M.

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Computational design of ligand-binding proteins with high affinity and selectivity

Christine E. Tinberg[1]*, Sagar D. Khare[1]†*, Jiayi Dou[2,3], Lindsey Doyle[4], Jorgen W. Nelson[5], Alberto Schena[6], Wojciech Jankowski[7], Charalampos G. Kalodimos[7], Kai Johnsson[6], Barry L. Stoddard[4] & David Baker[1,8]

The ability to design proteins with high affinity and selectivity for any given small molecule is a rigorous test of our understanding of the physiochemical principles that govern molecular recognition. Attempts to rationally design ligand-binding proteins have met with little success, however, and the computational design of protein–small-molecule interfaces remains an unsolved problem[1]. Current approaches for designing ligand-binding proteins for medical[2] and biotechnological uses rely on raising antibodies against a target antigen in immunized animals[3,4] and/or performing laboratory-directed evolution of proteins with an existing low affinity for the desired ligand[5–7], neither of which allows complete control over the interactions involved in binding. Here we describe a general computational method for designing pre-organized and shape complementary small-molecule-binding sites, and use it to generate protein binders to the steroid digoxigenin (DIG). Of seventeen experimentally characterized designs, two bind DIG; the model of the higher affinity binder has the most energetically favourable and pre-organized interface in the design set. A comprehensive binding-fitness landscape of this design, generated by library selections and deep sequencing, was used to optimize its binding affinity to a picomolar level, and X-ray co-crystal structures of two variants show atomic-level agreement with the corresponding computational models. The optimized binder is selective for DIG over the related steroids digitoxigenin, progesterone and β-oestradiol, and this steroid binding preference can be reprogrammed by manipulation of explicitly designed hydrogen-bonding interactions. The computational design method presented here should enable the development of a new generation of biosensors, therapeutics and diagnostics.

Computational design could provide a general approach for creating new small molecule binding proteins with rationally programmed specificities. Structural and biophysical characterization of previous computationally designed ligand-binding proteins revealed numerous discrepancies with the design models, however, and it was concluded that protein–ligand interaction design is an unsolved problem[1,8]. The lack of accuracy in programming protein–small-molecule interactions also contributes to low catalytic efficiencies of computationally designed enzymes[9–14]. The development of robust computational methods for the design of small-molecule-binding proteins with high affinity and selectivity would have wide-ranging applications.

We developed a computational method for designing ligand-binding proteins with three properties characteristic of naturally occurring binding sites: (1) specific energetically favourable hydrogen-bonding and van der Waals interactions with the ligand; (2) high overall shape complementarity to the ligand; and (3) structural pre-organization in the unbound protein state, which minimizes entropy loss upon ligand binding[15,16]. To program in defined interactions with the small molecule, disembodied binding sites are created by positioning amino acid side chains around the ligand in optimal orientations and then placed at geometrically compatible sites in a set of scaffold protein structures[17]. The surrounding side chain identities and conformations are then optimized to generate additional protein–ligand and buttressing protein–protein interactions (Fig. 1a). Designs with protein–small-molecule shape complementarity below those typical of native complexes[18] or having interface side chain conformations with low Boltzmann-weighted probabilities in the unbound state[16] are then discarded.

We used the method to design proteins that bind the steroid DIG (Supplementary Fig. 1), the aglycone of digoxin, a cardiac glycoside used to treat heart disease[19] and a non-radioactive biomolecular labelling reagent[20]. Anti-digoxigenin antibodies are administered to treat overdoses of digoxin, which has a narrow therapeutic window[21], and are used to detect biomolecules in applications such as fluorescence *in situ* hybridization[20]. We created idealized DIG-binding sites featuring hydrogen bonds from Tyr or His to the polar groups of DIG and hydrophobic packing interactions between Tyr, Phe or Trp and the steroid ring system (Fig. 1a). These interactions were embedded in designed binding sites with high shape complementarity to DIG, and 17 designs were selected for experimental characterization based on computed binding affinity, shape complementarity, and the extent of binding site pre-organization in the unbound state (Fig. 1b and Supplementary Tables 1 and 2).

Binding of the designed proteins to DIG was probed by yeast surface display[22] and flow cytometry using DIG-functionalized bovine serum albumin (DIG-BSA) or RNase (DIG-RNase). Designed proteins DIG5 and DIG10 bound to both labels (Fig. 1c and Supplementary Fig. 2), and binding was reduced to background levels when unlabelled DIG was added as a competitor (Fig. 1c and Supplementary Fig. 3). Fluorescence polarization measurements with purified proteins and Alexa488-fluorophore-conjugated DIG (DIG-PEG$_3$-Alexa488) indicated affinities in the low-to-mid micromolar range, with DIG10 binding more tightly (Fig. 2a, b). Isothermal titration calorimetry (ITC) measurements confirmed that the affinity of DIG10 for DIG is identical to that for DIG-PEG$_3$-Alexa488 (Fig. 2b, Supplementary Fig. 4 and Supplementary Table 3). The scaffold from which both DIG5 and DIG10 derive, a protein of unknown function from *Pseudomonas aeruginosa* (Protein Data Bank (PDB) accession code 1Z1S), does not bind to either label (Fig. 1c and Supplementary Fig. 3a) when expressed on the yeast surface or to DIG-PEG$_3$-Alexa488 in solution (Fig. 2a), suggesting that the binding activities of both proteins are mediated by the computationally designed interfaces. Indeed, substitution of small nonpolar residues in the binding pockets of DIG5 and DIG10 with arginines resulted in complete loss of binding, and mutation of the designed hydrogen-bonding tyrosine and histidine residues to

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[1] Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA. [2] Department of Bioengineering, University of Washington, Seattle, Washington 98195, USA. [3] Graduate Program in Biological Physics, Structure, and Design, University of Washington, Seattle, Washington 98195, USA. [4] Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109, USA. [5] Department of Genome Sciences, University of Washington, Seattle, Washington 98195, USA. [6] Ecole Polytechnique Fédérale de Lausanne, Institute of Chemical Sciences and Engineering, Institute of Bioengineering, National Centre of Competence in Research (NCCR) in Chemical Biology, 1015 Lausanne, Switzerland. [7] Department of Chemistry and Chemical Biology, Center for Integrative Proteomics Research, Rutgers University, Piscataway, New Jersey 08854, USA. [8] Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195, USA. †Present address: Department of Chemistry and Chemical Biology, Center for Integrative Proteomics Research, Rutgers University, Piscataway, New Jersey 08854, USA.

*These authors contributed equally to this work.

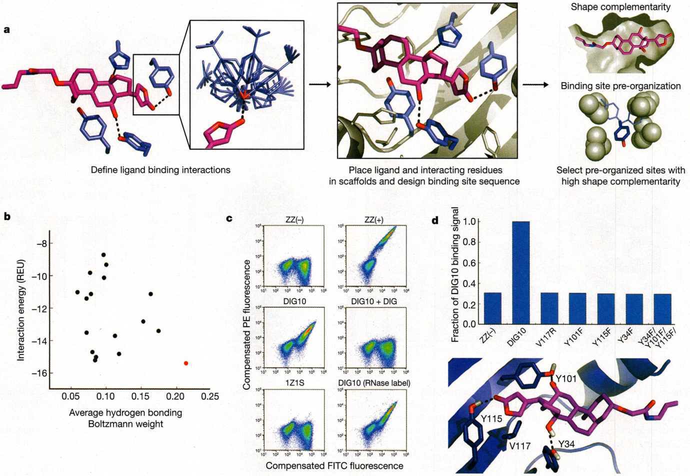

a
Define ligand binding interactions
Place ligand and interacting residues in scaffolds and design binding site sequence
Shape complementarity
Binding site pre-organization
Select pre-organized sites with high shape complementarity
b
Interaction energy (REU)
-8
-10
-12
-14
-16
0.05
0.10
0.15
0.20
0.25
Average hydrogen bonding Boltzmann weight
c
ZZ(–)
ZZ(+)
DIG10
DIG10 + DIG
1Z1S
DIG10 (RNase label)
Compensated PE fluorescence
Compensated FITC fluorescence
d
Fraction of DIG10 binding signal
1.0
0.8
0.6
0.4
0.2
0.0
ZZ(–)
DIG10
V117R
Y101F
Y115F
Y34F
Y34F/Y101F/Y115F/
Y101
Y115
V117
Y34

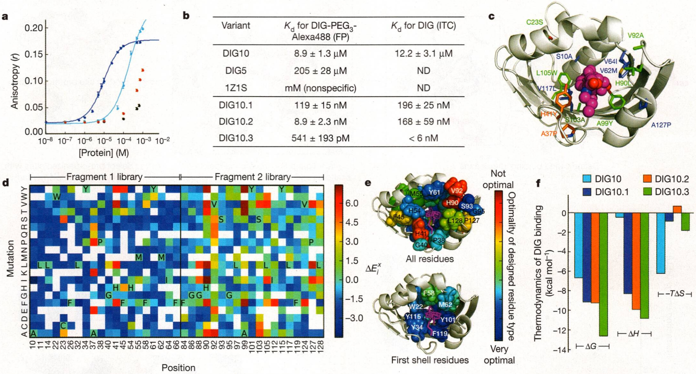
Figure 1 | Computational design methodology and experimental binding validation. **a**, Overview of the design procedure. **b**, Ranking of experimentally characterized DIG designs by computed ligand interaction energy (Rosetta energy units, REU) and average (geometric mean) side-chain Boltzmann weight of residues designed to hydrogen bond to DIG. DIG10 (red) scores the best by both metrics. **c**, Flow cytometric analysis of yeast cells expressing designed proteins. Yeast surface expression and DIG binding were probed by labelling with anti-c-Myc-fluorescein (FITC) and a mixture of biotinylated DIG-functionalized BSA and phycoerythrin (PE)–streptavidin, respectively. ZZ(−), negative control; ZZ(+), positive control; 1Z1S, original scaffold. **d**, On-yeast substitutions of DIG10-designed interface residues reduce binding (phycoerythrin) signals to background negative control levels. See figure legends in Supplementary Information for details.

phenylalanine reduced (DIG5) or abolished (DIG10) binding (Fig. 1d and Supplementary Fig. 5). Optimization of DIG10 by site-saturation mutagenesis and selections using yeast surface display and fluorescence-activated cell sorting (FACS) identified several small-to-large hydrophobic amino acid changes that increase binding affinity 75-fold through enhanced binding enthalpy, yielding DIG10.1 (Fig. 2b, c, f, Supplementary Figs 4, 6−8 and Supplementary Table 3).

To provide feedback for improving the overall design methodology and to evaluate the contribution of each residue in the DIG10.1-binding site, we used next-generation sequencing to generate a comprehensive binding fitness map[23–25]. A library of variants with approximately 1–3 substitutions at 39 designed interface positions in DIG10.1 was generated using doped oligonucleotide mutagenesis, displayed on yeast, and subjected to selections using a monovalent DIG-PEG$_3$-biotin conjugate (Supplementary Fig. 9). Variants with increased affinity for DIG were selected by FACS, and next-generation sequencing was used to quantify the frequency of every single point mutation in the unselected and selected populations. A large majority of the interrogated variants were depleted in the selected population relative to the unselected input library, suggesting that most of the DIG10.1-binding site residues are optimal for binding (Fig. 2d, e and Supplementary Fig. 10). In particular, mutation of the three designed hydrogen-bonding residues, Tyr 34, Tyr 101 and Tyr 115, to any other amino acid was disfavoured. Several large hydrophobic residues that pack against the ligand in the computational model are also functionally optimal (for example, Phe 66 and Phe 119). Besides Ala 99, which contacts DIG directly, most of the observed mutations that improve binding are located in the second coordination shell of the ligand and fall into two categories: (1) protein core substitutions tolerating mutation to chemically similar amino acids (for example, Leu 105 and Cys 23), and (2) solvent-exposed loop residues having high sequence entropy (for example, His 90 and Val 92). The best clone obtained from sorting the library to homogeneity, DIG10.2, contains two of the most highly enriched mutations, Ala37Pro and His41Tyr (Fig. 2b, c and Supplementary Figs 4, 6, 8 and 11).

To increase binding affinity further, we constructed a library in which the residues at 11 positions that acquired beneficial substitutions in the deep sequencing experiment were varied in combination to allow for non-additive effects. Selections led to DIG10.3 (Supplementary Figs 4, 6, 8 and 12), which binds DIG and its cardiac glycoside derivative digoxin with picomolar affinity (Fig. 2b, Supplementary Fig. 13 and Supplementary Table 4), rivalling the affinities of anti-digoxin antibody therapeutics[21] and an evolved single-chain variable anti-digoxin antibody fragment[7]. Fluorescence-polarization-based affinity measurements of DIG10.3 and Tyr knockouts suggest that the designed hydrogen bonds each contribute ~2 kcal mol^{-1} to binding energy (Supplementary Table 5 and Supplementary Fig. 8).

The crystal structures of DIG10.2 and DIG10.3 in complex with DIG were solved to 2.05 Å and 3.2 Å resolution, respectively (Fig. 3a, b and



Variant	K_d for DIG-PEG$_3$-Alexa488 (FP)	K_d for DIG (ITC)
DIG10	8.9 ± 1.3 μM	12.2 ± 3.1 μM
DIG5	205 ± 28 μM	ND
1Z1S	mM (nonspecific)	ND
DIG10.1	119 ± 15 nM	196 ± 25 nM
DIG10.2	8.9 ± 2.3 nM	168 ± 59 nM
DIG10.3	541 ± 193 pM	< 6 nM

Figure 2 | Binding characterization and affinity maturation. **a**, Equilibrium fluorescence anisotropy of DIG-PEG$_3$-Alexa488 mixed with purified DIG10 (blue), DIG5 (cyan), 1Z1S scaffold (black) and BSA (red). Error bars represent s.d. for at least three independent measurements. **b**, Dissociation constants (K_d values) of designs. Differences in fluorescence polarization (FP)- and isothermal titration calorimetry (ITC)-derived K_d values probably result from enhanced interactions with the linker of DIG-PEG$_3$-Alexa488 used in the fluorescence polarization experiments. ND, not determined. **c**, Mutations identified during affinity maturation to generate DIG10.1 (blue), DIG10.2 (orange) and DIG10.3 (green) mapped onto the computational model of DIG10.3. **d**, Fitness landscape of DIG10.1 showing the effects of single amino acid substitutions on binding (ΔE_i^x; see equation (1) in Methods). Red and blue indicate enrichment and depletion, respectively. The original DIG10.1 amino acid at each position is indicated in bold. White indicates mutations for which there were not enough sequences in the unselected library to make a statistically significant conclusion about function. **e**, The optimality of each initial DIG10.1 residue type mapped onto the computational model of DIG10.1. **f**, DIG binding thermodynamic parameters determined by ITC. ΔG, free binding energy; ΔH, binding enthalpy; −TΔS, binding entropy. See figure legends in Supplementary Information for details.

Supplementary Figs 14–19). The structure of DIG10.2 bound to DIG shows atomic-level agreement with the design model (all-atom root mean squared deviation (r.m.s.d.) = 0.54 Å; Fig. 3c). The ligand–protein interface has high shape complementarity (S_c = 0.66) and no water molecules are observed in the binding pocket. The DIG binding mode is nearly identical in the X-ray structure and the computational model, with an average r.m.s.d. of 0.99 Å for all ligand heavy atoms (Fig. 3d). As designed, Tyr 34, Tyr 101 and Tyr 115 hydrogen bond with O3, O2 and O1 of DIG, respectively. Tyr 41, a residue identified during affinity maturation, forms a weak hydrogen bond with the terminal hydroxyl group of DIG (O5) (Supplementary Fig. 16). Of 27 non-glycine/alanine residues within ~10 Å of the ligand, 21 adopt the computationally designed conformations (Supplementary Fig. 17), including Tyr 101 and Tyr 115 (in chain B) as well as the first-shell packing residues Trp 22, Phe 58 and Phe 119. The structure of DIG10.3 bound to DIG (Supplementary Fig. 18) also agrees closely with the design model (r.m.s.d. = 0.68 Å).

We assessed the binding specificity of DIG10.3 by determining affinities for a series of related steroids by equilibrium competition fluorescence polarization. Digitoxigenin, progesterone and β-oestradiol bind less tightly to DIG10.3 than DIG (Fig. 4a, b and Supplementary Table 4). The magnitudes of the affinity decreases are consistent with the loss of one, two and three hydrogen bonds, respectively (assuming ~1.8 kcal mol^{-1} per hydrogen bond[26]), suggesting that these compounds bind in the same orientation as DIG. We next investigated whether the observed steroid selectivity could be reprogrammed by mutagenesis of the key hydrogen-bonding tyrosines. The variants Tyr101Phe, Tyr34Phe and Tyr34Phe/Tyr99Phe/Tyr101Phe show clear preferences for more hydrophobic steroids in a predictable manner that depends on the hydrogen-bonding capabilities of both the protein and the steroid. Tyr101Phe eliminates the DIG-specific hydrogen bond with DIG O2 and provides a more hydrophobic environment that favours the other three steroids (Fig. 4c). Tyr34Phe removes a hydrogen bond common to DIG and digitoxigenin, thus enhancing the preference for progesterone (Fig. 4d). Tyr34Phe/Tyr99Phe/Tyr101Phe has decreased affinity for DIG and increased affinity for the more hydrophobic steroids (Fig. 4e). These results confirm that the selectivity of DIG10.3 for DIG is conferred through the designed hydrogen-bonding interactions and demonstrate how this feature can be programmed using positive design alone through the explicit placement of designed polar and hydrophobic interactions.

Comparison of the properties of successful and unsuccessful designs provides a test of the hypotheses underlying the design methodology. Although all 17 designed proteins had high computed shape complementarity to DIG by construction, the DIG10 design, which had the highest affinity for DIG, had the most favourable computed protein–ligand interaction energy and was predicted to have the most pre-organized binding site (Fig. 1b and Supplementary Table 6), suggesting that these attributes should continue to be the focus of future design methodology development. One potential avenue for obtaining more favourable interaction energies would be incorporating backbone flexibility during design to achieve more tightly packed binding sites: the fact that substitution of small hydrophobic interface residues to larger ones increased binding affinity indicates that the original DIG10 design was under-packed.

The binding fitness landscape in combination with the X-ray co-crystal structures highlight the importance of second shell interactions

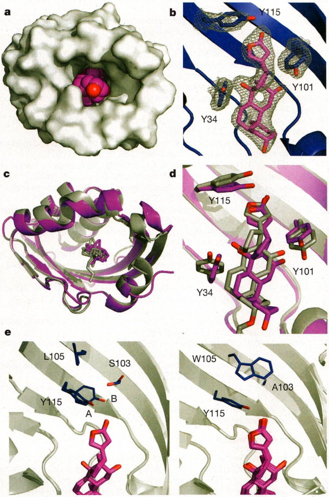


Figure 3 | Crystal structures of DIG10.2–DIG and DIG10.3–DIG. a, Surface representation of the DIG10.2–DIG complex. DIG is shown in magenta. DIG10.2 is a dimer and crystallized with four copies in the asymmetric unit. **b**, DIG10.2–DIG $2F_o - F_c$ omit map electron density showing DIG and interacting tyrosines contoured at 1.0σ. **c**, Backbone superposition of the crystal structure of DIG10.2–DIG (magenta) with the computational model (grey). **d**, DIG10.2–DIG-binding site backbone superposition. **e**, Comparison of an overlay of the four DIG10.2-DIG crystallographic copies (left) with that of DIG10.3-DIG chains A, B, C, H and I (right; the density was poorly resolved for the other four chains). DIG10.2 Tyr 115 conformation A has a more canonical hydrogen-bonding geometry than conformation B, and in DIG10.3 Tyr 115 is locked into conformation A by Trp 105.

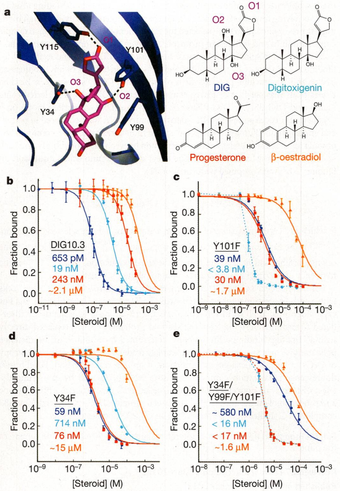


Figure 4 | Steroid binding selectivity. a, X-ray crystal structure of the DIG10.3–DIG complex (left) and chemical structures of related steroids (right). **b**, Steroid selectivity profile of DIG10.3 determined by measuring the equilibrium effects of unlabelled steroids on the anisotropy of the DIG-PEG$_3$-Alexa488–DIG10.3 complex. Solid lines represent fits of the data to a competitive binding model and error bars indicate s.d. for at least three independent measurements. Inhibition constants (K_i values, coloured numbers) were estimated from inhibitory half-maximal concentrations (IC$_{50}$ values) obtained from the fits. **c**, Steroid selectivity profile of DIG10.3(Tyr101Phe). Dashed lines show qualitative assessments of the inhibitory effects for cases in which the data could not be fit due to overly strong inhibition (see Supplementary Methods). **d**, Steroid selectivity profile of DIG10.3(Tyr34Phe). **e**, Steroid selectivity profile of DIG10.3(Tyr34Phe/Tyr99Phe/Tyr101Phe).

in stabilizing binding competent conformations. For example, the enriched substitution Leu105Trp (Fig. 2d) causes the adjacent Tyr 115 side chain, which shows conformational variability in DIG10.2, to adopt a single conformation in DIG10.3 that makes a more canonical hydrogen bond to DIG than that of the DIG10.2 alternative state (Fig. 3e and Supplementary Fig. 19). The calculated side chain pre-organization of all three hydrogen-bonding tyrosine residues increases from DIG10.2 to DIG10.3 (Supplementary Table 7), suggesting that the increased affinity may arise in part from a higher proportion of the binding competent conformation of apo-DIG10.3 (refs 15, 27). Indeed, ITC studies confirm that entropic as well as enthalpic factors contribute to the enhanced binding affinity of DIG10.3 (Fig. 2f and Supplementary Table 3). Similarly, reduced backbone conformational entropy is probably responsible for the increased fitness of substitutions increasing β-sheet propensity at inter-strand loop positions 90 and 92 (Fig. 2d). That flexibility is selected against during affinity maturation suggests that maximizing the free-energy gap between binding-competent and alternative states of the binding site[28] by explicitly designing second shell interactions to buttress side chains making key ligand contacts should help to achieve high affinity in the next generation of computationally designed ligand-binding proteins.

The binding affinity of DIG10.3 is similar to those of anti-digoxin antibodies[21], and because it is stable for extended periods (>3 months) at ambient temperatures (Supplementary Fig. 20) and can be expressed at high levels in bacteria, it could provide a more cost-effective alternative for biotechnological and for therapeutic purposes if it can be made compatible with the human immune response. With continued improvement in the methodology and feedback from experimental results, computational protein design should provide an increasingly powerful approach to creating small molecule receptors for synthetic biology, therapeutic scavengers for toxic compounds, and robust binding domains for diagnostic devices.

METHODS SUMMARY

Design calculations were performed using RosettaMatch[17] to incorporate five predefined interactions to DIG into a set of 401 scaffolds. RosettaDesign[29] was then used to optimize each binding site sequence for maximal ligand-binding affinity. Designs having low interface energy, high shape complementarity, and high binding site pre-organization were selected for experimental characterization. Example command lines and full design protocols are given in the Supplementary Data.

Designs were displayed on the surface of yeast strain EBY100 and examined for binding to a mixture of 2.7 μM biotinylated DIG-conjugated BSA or DIG-conjugated RNase and streptavidin–phycoerythrin on an Accuri C6 flow cytometer. Binding clones from yeast-surface displayed libraries based on DIG10 were selected using highly avid DIG-BSA or DIG-RNase or monovalent DIG-conjugated biotin on a Cytopeia inFlux cell sorter. DIG10.1-derived library DNA was sequenced in paired-end mode on an Illumina MiSeq. For single mutations having ≥7 counts in the original input library, a relative enrichment ratio between the input library and each selected library was calculated[23–25]. The effect of each amino acid substitution on binding, ΔE_i^x, was computed with equation (1),

$$\Delta E_i^x = \log_2\left(\frac{f_i^{x,\mathrm{sel}}}{f_i^{x,\mathrm{unsel}}}\right) - \log_2\left(\frac{f_i^{\mathrm{orig,sel}}}{f_i^{\mathrm{orig,unsel}}}\right) \qquad (1)$$

in which $f_i^{x,\mathrm{sel}}$ is the frequency of mutation x at position i in the selected population, $f_i^{x,\mathrm{unsel}}$ is the frequency in the unselected population, $f_i^{\mathrm{orig,sel}}$ is the frequency of the original amino acid at position i in the selected population, and $f_i^{\mathrm{orig,unsel}}$ is the frequency of the original amino acid in the unselected population.

For biochemical assays, proteins were expressed in *E. coli* Rosetta 2 (DE3) cells with a carboxy-terminal tobacco etch virus (TEV) protease-cleavable His_6 tag. For crystallographic analysis of DIG10 variants, a 12-amino-acid structurally disordered C terminus deriving from the scaffold protein 1Z1S was replaced directly with a His_6 tag. Binding affinities were determined by equilibrium fluorescence polarization[30] on a SpectraMax M5e microplate reader by monitoring the anisotropy of DIG-conjugated Alexa488 as a function of protein concentration. Equilibrium fluorescence polarization competition assays were performed by examining the effect of increasing concentrations of unlabelled DIG, digitoxigenin, progesterone and β-oestradiol on the anisotropy of designed protein−DIG-conjugated Alexa488 complex. ITC studies were performed on an iTC200 microcalorimeter.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions C.E.T., S.D.K. and D.B. designed the research. S.D.K., C.E.T. and J.D. performed computations. S.D.K. wrote program code. C.E.T. experimentally characterized the designs, constructed libraries, performed affinity maturation and deep sequencing selections, and conducted binding and biochemical studies of DIG10. J.D. characterized DIG5. J.W.N. analysed deep sequencing data. C.E.T. and J.D. prepared protein samples for crystallographic analysis. L.D. and B.S. crystallized the protein samples and analysed crystallographic data. A.S. and K.J. synthesized DIG-PEG_3-biotin and DIG-PEG_3-Alexa488. C.E.T. prepared protein samples for ITC studies, and W.J. and C.G.K. performed ITC experiments and analysed ITC data. C.E.T., S.D.K. and D.B. analysed data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Author Information The crystal structures of DIG10.2 and DIG10.3 bound to DIG have been deposited in the RCSB Protein Data Bank under the accession codes 4J8T (DIG10.2) and 4J9A (DIG10.3). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.B. (dabaker@u.washington.edu).

LETTER

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De novo mutations in epileptic encephalopathies

Epi4K Consortium* & Epilepsy Phenome/Genome Project*

Epileptic encephalopathies are a devastating group of severe childhood epilepsy disorders for which the cause is often unknown[1]. Here we report a screen for *de novo* mutations in patients with two classical epileptic encephalopathies: infantile spasms ($n = 149$) and Lennox–Gastaut syndrome ($n = 115$). We sequenced the exomes of 264 probands, and their parents, and confirmed 329 *de novo* mutations. A likelihood analysis showed a significant excess of *de novo* mutations in the ~4,000 genes that are the most intolerant to functional genetic variation in the human population ($P = 2.9 \times 10^{-3}$). Among these are *GABRB3*, with *de novo* mutations in four patients, and *ALG13*, with the same *de novo* mutation in two patients; both genes show clear statistical evidence of association with epileptic encephalopathy. Given the relevant site-specific mutation rates, the probabilities of these outcomes occurring by chance are $P = 4.1 \times 10^{-10}$ and $P = 7.8 \times 10^{-12}$, respectively. Other genes with *de novo* mutations in this cohort include *CACNA1A*, *CHD2*, *FLNA*, *GABRA1*, *GRIN1*, *GRIN2B*, *HNRNPU*, *IQSEC2*, *MTOR* and *NEDD4L*. Finally, we show that the *de novo* mutations observed are enriched in specific gene sets including genes regulated by the fragile X protein ($P < 10^{-8}$), as has been reported previously for autism spectrum disorders[2].

Genetics is believed to have an important role in many epilepsy syndromes; however, specific genes have been discovered in only a small proportion of cases. Genome-wide association studies for both focal and generalized epilepsies have revealed few significant associations, and rare copy number variants explain only a few per cent of cases[3–6]. An emerging paradigm in neuropsychiatric disorders is the major impact that *de novo* mutations have on disease risk[7,8]. We searched for *de novo* mutations associated with epileptic encephalopathies, a heterogeneous group of severe epilepsy disorders characterized by early onset of seizures with cognitive and behavioural features associated with ongoing epileptic activity. We focused on two 'classical' forms of epileptic encephalopathies: infantile spasms and Lennox–Gastaut syndrome, recognizing that some patients with infantile spasms progress to Lennox–Gastaut syndrome.

Exome sequencing of 264 trios (Methods) identified 439 putative *de novo* mutations. Sanger sequencing confirmed 329 *de novo* mutations (Supplementary Table 2), and the remainder were either false positives, a result of B-cell immortalization, or in regions where the Sanger assays did not work (Supplementary Table 3).

Across our 264 trios, we found nine genes with *de novo* single nucleotide variant (SNV) mutations in two or more probands (*SCN1A*, $n = 7$; *STXBP1*, $n = 5$; *GABRB3*, $n = 4$; *CDKL5*, $n = 3$; *SCN8A*, $n = 2$; *SCN2A*, $n = 2$; *ALG13*, $n = 2$; *DNM1*, $n = 2$; and *HDAC4*, $n = 2$). Of these, *SCN1A*, *STXBP1*, *SCN8A*, *SCN2A* and *CDKL5* are genes that have a previously established association with epileptic encephalopathy[9–14]. To assess whether the observations in the other genes implicate them as risk factors for epileptic encephalopathies, we determined the probability of seeing multiple mutations in the same gene given the sequence-specific mutation rate, size of the gene, and the number and gender of patients evaluated in this study (Methods). The number of observed *de novo* mutations in *HDAC4* and *DNM1* are not yet significantly greater than the null expectation. However, observing four unique *de novo* mutations in *GABRB3* and two identical *de novo* mutations in *ALG13* were found to be highly improbable (Table 1 and Fig. 1). We performed the same calculations on all of the genes with multiple *de novo* mutations observed in 610 control trios and found no genes with a significant excess of *de novo* mutations (Supplementary Table 4). Although mutations in *GABRB3* have previously been reported in association with another type of epilepsy[15], and *in vivo* mouse studies suggest that *GABRB3* haploinsufficiency is one of the causes of epilepsy in Angelman's syndrome[16], our observations implicate it, for the first time, as a single-gene cause of epileptic encephalopathies and provide the strongest evidence to date for its association with any epilepsy. Likewise, *ALG13*, an X-linked gene encoding a subunit of the uridine diphosphate-*N*-acetylglucosamine transferase, was previously shown to carry a novel *de novo* mutation in a male patient with a severe congenital glycosylation disorder with microcephaly, seizures and early lethality[17]. Furthermore, the same *ALG13* *de novo* mutation identified in this study was observed as a *de novo* mutation in an additional female patient with severe intellectual disability and seizures[18].

Each trio harboured on average 1.25 confirmed *de novo* mutations, with 181 probands harbouring at least one. Considering only *de novo* SNVs, each trio harboured on average 1.17 *de novo* mutations (Supplementary Fig. 1). Seventy-two per cent of the confirmed *de novo* SNV mutations were missense and 7.5% were putative loss-of-function (splice donor, splice acceptor, or stop-gain mutations). Compared to rates of these classes of mutations previously reported in controls (69.4% missense and 4.2% putative loss-of-function mutations)[2,19,20], we observed a significant excess of loss-of-function mutations in patients with infantile spasms and Lennox–Gastaut syndrome (exact binomial $P = 0.01$), consistent with data previously reported in autism spectrum disorder[2,8,19,20].

A framework was recently established for testing whether the distribution of *de novo* mutations in affected individuals differs from the general population[8]. Here, we extend the simulation-based approach of ref. 8 by developing a likelihood model that characterizes this effect and describes the distribution of *de novo* mutations among affected individuals in terms of the distribution in the general population, and a set of parameters describing the genetic architecture of the disease. These parameters include the proportion of the exome sequence that can carry disease-influencing mutations (η) and the relative risk (γ) of the mutations (Supplementary Methods). Consistent with what was reported in autism spectrum disorder[8], we found no significant deviation in the overall distribution of mutations from expected ($\gamma = 1$ and/or $\eta = 0$). It is, however, now well established that some genes tolerate protein-disrupting mutations without apparent adverse phenotypic consequences, whereas others do not[21]. To take this into account, we used a simple scoring system that uses polymorphism data in the human population to assign a tolerance score to every considered gene (Methods). We then found that genes with a known association with epileptic encephalopathy rank among the most intolerant genes using this scheme (Supplementary Table 8). We therefore evaluated the distribution of *de novo* mutations within these 4,264 genes that are within the 25th percentile for intolerance and found a significant shift from the null distribution ($P = 2.9 \times 10^{-3}$). The maximum likelihood estimates of η (percentage of intolerant genes involved in epileptic encephalopathies) was 0.021 and γ (relative risk) was 81, indicating that there are 90 genes among the intolerant genes

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*A list of authors and affiliations appears at the end of the paper.

Table 1 | Probability of observing the reported number of *de novo* mutations by chance in genes recurrently mutated in this cohort

Gene	Chromosome	Average effectively captured length (bp)	Weighted mutation rate	*De novo* mutation number	*P* value†	
SCN1A	2	6,063.70	1.61×10^{-4}	5‡	1.12×10^{-9}	***
STXBP1	9	1,917.51	6.44×10^{-5}	5	1.16×10^{-11}	***
GABRB3	15	1,206.86	3.78×10^{-5}	4	4.11×10^{-10}	***
CDKL5	X	2,798.38	5.44×10^{-5}	3	4.90×10^{-7}	**
ALG13§	X	475.05	1.03×10^{-5}	2	7.77×10^{-12}	***
DNM1	9	2,323.37	9.10×10^{-5}	2	2.84×10^{-4}	
HDAC4	2	2,649.82	1.16×10^{-4}	2	4.57×10^{-4}	
SCN2A§	2	5,831.21	1.52×10^{-4}	2	1.14×10^{-9}	***
SCN8A	12	5,814.48	1.64×10^{-4}	2	9.14×10^{-4}	

† Adjusted α is equivalent to $0.05/18{,}091 = 2.76 \times 10^{-6}$ (*), $0.01/18{,}091 = 5.53 \times 10^{-7}$ (**) and $0.001/18{,}091 = 5.53 \times 10^{-8}$ (***).

‡ Counts exclude three additional patients with an indel or splice site mutation as these are not accounted for in the mutability calculation.

§ Two *de novo* mutations occur at the same position. The probability of these special cases obtain $P = 7.77 \times 10^{-12}$ and $P = 1.14 \times 10^{-9}$ for *ALG13* and *SCN2A*, respectively (Methods).

that can confer risk of epileptic encephalopathies and that each mutation carries substantial risk. We also found that putatively damaging *de novo* variants in our cohort are significantly enriched in intolerant genes compared with control cohorts (Supplementary Methods).

We next evaluated whether the *de novo* mutations were drawn preferentially from six gene sets (Methods and Supplementary Table 10), including ion channels[22], genes known to cause monogenic disorders with seizures as a phenotypic feature[23], genes carrying confirmed *de novo* mutations in patients with autism spectrum disorder[2,8,19,20] and in patients with intellectual disability[18,24], and FMRP-regulated genes. Taking into account the size of regions with adequate sequencing coverage to detect a *de novo* mutation (Methods), we found significant over-representation for all gene lists in our data (Supplementary Table 10), and no over-representation in controls[2,19,20,24].

To determine possible interconnectivity among the genes carrying a *de novo* mutation, we performed a protein–protein interaction analysis and identified a single network of 71 connected proteins (Fig. 2 and Supplementary Fig. 7). These 71 proteins include six encoded by OMIM reported epileptic encephalopathy genes (http://www.omim.org/) where we identified one or more *de novo* mutations among the epileptic encephalopathy patients in this study. Genes in this protein–protein network were also found to have a much greater probability of overlap with the autism spectrum disorder[2,8,19,20] and severe intellectual disability disorder[18,24] exome sequencing study genes, and with FMRP-associated genes, than genes not in this network (Supplementary Table 11).

In support of a hypothesis that individual rare mutations in different genes may converge on biological pathways, we draw attention to six mutations that all affect subunits of the GABA (γ-aminobutyric acid) ionotropic receptor (four in *GABRB3*, and one each in *GABRA1* and *GABRB1*), and highlight two interactions: *HNRNPU* interacting with *HNRNPH1*, and *NEDD4L* (identified here) binding to *TNK2*, a gene previously implicated in epileptic encephalopathies[25] (Fig. 2). Although the *HNRNPU* mutation observed here is a small insertion/deletion variant (indel) in a splice acceptor site, and therefore probably results in a modified protein, the *HNRNPH1 de novo* mutation is synonymous and thus of unknown functional significance (Supplementary Table 2). Notably, a minigene experiment indicates that this synonymous mutation induces skipping of exon 12 (Supplementary Methods).

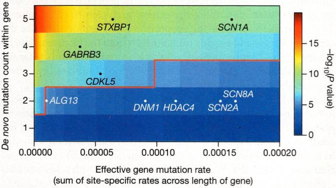

Figure 1 | Heat map illustrating the probability of observing the specified number of *de novo* mutations in genes with the specified estimated mutation rate. The number of *de novo* mutations required to achieve significance is indicated by the solid red line. The superimposed dots reflect positions of all genes found to harbour multiple *de novo* mutations in our study. *GABRB3*, *SCN1A*, *CDKL5* and *STXBP1* have significantly more *de novo* mutations than expected. The positions indicated for *ALG13* and *SCN2A* reflect only the fact that there are two mutations observed, not that there are two mutations affecting the same site (Methods).

Evaluation of the clinical phenotypes among patients revealed significant genetic heterogeneity underlying infantile spasms and Lennox–Gastaut syndrome, and begins to provide information about the range of phenotypes associated with mutations in specific genes (Supplementary Table 13). We identified four genes—*SCN8A*, *STXBP1*, *DNM1* and *GABRB3*—with *de novo* mutations in both patients with infantile spasms and patients with Lennox–Gastaut syndrome. Although infantile spasms may progress to Lennox–Gastaut syndrome, in three of these cases the patients with Lennox–Gastaut syndrome did not initially present with infantile spasms, indicating phenotypic heterogeneity associated with mutations in these genes yet supporting the notion of shared genetic susceptibility. Notably, in multiple patients we identified *de novo* mutations in genes previously implicated in other neurodevelopmental conditions, and in some cases with very distinctive clinical presentations (Supplementary Table 12). Most notably, we found a *de novo* mutation in *MTOR*, a gene recently found to harbour a causal variant in mosaic form in a case with hemimegalencephaly[26]. Our patient however showed no detectable structural brain malformation. Similarly, we found one patient with a *de novo* mutation in *DCX* and another with a *de novo* mutation in *FLNA*, previously associated with lissencephaly and periventricular nodular heterotopia, respectively[27,28]; neither patient had cortical malformations detected on magnetic resonance imaging.

In addition to *de novo* variants, we also screened for highly penetrant genotypes by identifying variants that create newly homozygous, compound heterozygous, or hemizygous genotypes in the probands that are not seen in parents or controls (Supplementary Methods). No inherited variants showed significant evidence of association. Additional studies evaluating a larger number of epileptic encephalopathy patients will be required to establish the role of inherited variants in the disease risk associated with infantile spasms and Lennox–Gastaut syndrome.

We have identified novel *de novo* mutations implicating at least two genes for epileptic encephalopathies, and also describe a genetic architecture that strongly suggests that we have identified additional causal mutations in genes intolerant to functional variation. Given that our sample size already shows many genes with recurrent mutations, it is clear that even modest increases in sample sizes will confirm many new genes now seen in only one of our trios. Our results also emphasize that it may be difficult to predict with confidence the responsible gene, even among known genes, based upon clinical presentation. This makes it clear that the future of genetic diagnostics in epileptic encephalopathies will need to focus on the genome as a whole as opposed to single genes or even gene panels. In particular, several of the genes with *de novo* mutations in our cohort have also been identified in patients with

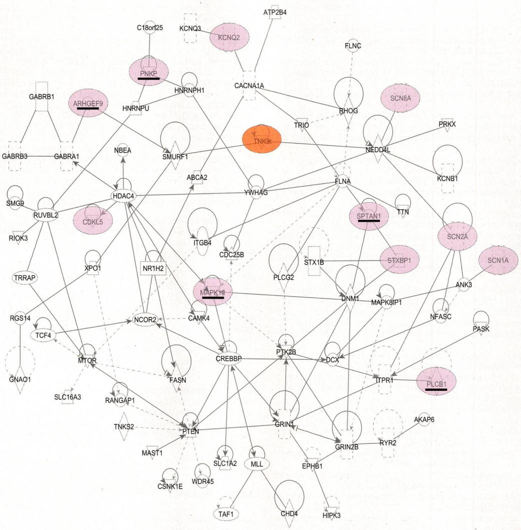
ATP2B4
C18orf25
KCNQ3
KCNQ2
FLNC
PNKP
HNRNPH1
CACNA1A
SCN8A
GABRB1
ARHGEF9
HNRNPU
RHOG
TRIO
PRKX
TNK2
NEDD4L
NBEA
SMURF1
GABRB3
GABRA1
KCNB1
ABCA2
FLNA
HDAC4
YWHAG
SMG9
RUVBL2
CDKL5
SPTAN1
TTN
RIOK3
ITGB4
SCN2A
SCN1A
CDC25B
STX1B
STXBP1
XPO1
NR1H2
PLCG2
TRRAP
ANK3
MAPK10
DNM1
MAPK8IP1
RGS14
CAMK4
NCOR2
NFASC
PASK
TCF4
MTOR
CREBBP
PTK2B
DCX
GNAO1
FASN
ITPR1
PLCB1
SLC16A3
RANGAP1
AKAP6
GRIN1
TNKS2
PTEN
RYR2
GRIN2B
MAST1
SLC1A2
MLL
EPHB1
CSNK1E
WDR45
TAF1
CHD4
HIPK3

Figure 2 | A protein–protein interaction network of genes with *de novo* mutations found in infantile spasms and Lennox–Gastaut syndrome patients studied. The geometric shapes reflect differing protein roles, as defined by ingenuity pathway analysis (Ingenuity Systems): enzyme, rhombus; ion channel, vertical rectangle; kinase, inverted triangle; ligand-dependent nuclear receptor, horizontal rectangle; phosphatase, triangle; transcription regulator, horizontal oval; transmembrane receptor, vertical oval; transporter, trapezoid; and unknown, circle. Six of the genes found to harbour *de novo* mutations in an infantile spasms or Lennox–Gastaut syndrome patient are known OMIM genes associated with epileptic encephalopathy (shaded circles). Five additional known OMIM genes associated with epileptic encephalopathy that were not found to be mutated in the 264 epileptic encephalopathy patients, but are involved in this network, are also shown (shaded circles with the gene underlined). The previously identified severe infantile epilepsy gene *TNK2* is superimposed into this network (red circle).

intellectual disability or autism spectrum disorder. Finally, and perhaps most importantly, this work suggests a clear direction for both drug development and treatment personalization in the epileptic encephalopathies, as many of these mutations seem to converge on specific biological pathways.

METHODS SUMMARY

All probands and family members were collected as part of the Epilepsy Phenome/Genome Project (EPGP) cohort[29] (Supplementary Table 1). Detailed inclusion and exclusion criteria are provided in Methods. Patient collection and sharing of specimens for research were approved by site-specific Institutional Review Boards.

We sequenced the exome of each trio, from DNA derived from primary cells ($n = 224$ trios) or from lymphoblastoid cell lines (LCLs) in one or more family members ($n = 40$ trios), using the TruSeq Exome Enrichment kit (Illumina). We aligned samples and called variants using established algorithms (Methods) and identified candidate *de novo* variants at sites included in the exons or splice sites of the consensus coding sequence (CCDS) as those called in the affected child and absent in both parents, despite each parent having at least tenfold coverage at the site. Variants created by the *de novo* mutation also had to be absent in our internal controls ($n = 436$), as well as the approximately 6,500 samples represented in the Exome Variant Server (http://evs.gs.washington.edu/EVS), and had to pass visual inspection of alignment quality. Candidate *de novo* mutations were confirmed to be *de novo* mutations using Sanger sequencing. In all cases, primary DNA from the proband was used for the Sanger confirmation so that mutations appearing in the transformation process for the 40 trios sequenced from LCLs would be eliminated.

To determine whether our list of *de novo* mutations was preferentially located in genes contained in the six gene lists we calculated the proportion of CCDS *de novo* mutation opportunity space for each list (Additional Methods). A binomial probability calculation was used to determine whether the *de novo* mutations in CCDS transcripts identified in this cohort of epileptic encephalopathy patients were selectively enriched within the coding sequence of genes within a particular gene list (Supplementary Table 10).

Ingenuity Pathway Analysis (Ingenuity Systems) was used to assess the connectivity of proteins harbouring a *de novo* mutation.

Full Methods and any associated references are available in the online version of the paper.

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Author contributions Initial design of EPGP: B.K.A., O.D., D.D., M.P.E., R.Kuz., D.H.L., R.O., E.H.S. and M.R.W. EPGP patient recruitment and phenotyping: B.A.-K., J.F.B., S.F.B., G.C., D.C., P.Cr., O.D., D.D., M.F., N.B.F., D.F., E.B.G., T.G., S.G., S.R.H., J.H., S.L.H., H.E.K., R.C.K., E.H.K., R.Kup., R.Kuz., D.H.L., S.M.M., P.V.M., E.J.N., J.M.Pao., J.M.Par., K.P., A.P., I.E.S., J.J.S., R.S., J.Si., M.C.S., L.L.T., A.V., E.P.G.V., G.K.V.A., J.L.W. and P.W.-W. Phenotype data analysis: B.A.-K., B.K.A., A.B., G.C., O.D., D.D., J.F., T.G., S.J., A.K., R.C.K., R.Kuz., D.H.L., R.O., J.M.Pao., A.P., I.E.S., R.A.S., E.H.S., J.J.S., J.Su., P.W.-W. and M.R.W. Initial design of Epi4K: S.F.B., P.Co., N.D., D.D., E.E.E., M.P.E., T.G., D.B.G., E.L.H., M.R.J., R.Kuz., D.H.L., A.G.M., H.C.M., T.J.O., R.O., A.P., I.E.S. and E.H.S. Epileptic encephalopathy phenotyping strategy: S.F.B., P.Co., D.D., R.Kuz., D.H.L., R.O., I.E.S. and E.H.S. Encephalopathy phenotyping: D.D., K.B.H., M.R.Z.M., H.C.M., A.P., I.E.S., E.H.S. and C.J.Y. Sequence data analysis and statistical interpretation: A.S.A., D.B.G., Y.Ha., E.L.H., S.E.N., S.P., E.K.R. and E.H.S. Functional evaluation of identified mutations: D.B.G., E.L.H., Y.Hi. and Y.-F.L. Writing of manuscript: A.S.A., S.F.B., D.D., D.B.G., Y.Ha., E.L.H., M.R.J., D.H.L., H.C.M., R.O., A.P., S.P., E.K.R., I.E.S. and E.H.S.

Author Information Exome sequence data will be available in dbGAP (Epi4K: Gene Discovery in 4,000 Epilepsy Genomes). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to Epi4K (epi4k@duke.edu).

Epi4K Consortium

Andrew S. Allen[1], Samuel F. Berkovic[2], Patrick Cossette[3], Norman Delanty[4], Dennis Dlugos[5], Evan E. Eichler[6], Michael P. Epstein[7], Tracy Glauser[8], David B. Goldstein[9], Yujun Han[9], Erin L. Heinzen[9], Yuki Hitomi[9], Katherine B. Howell[10], Michael R. Johnson[11], Ruben Kuzniecky[12], Daniel H. Lowenstein[13], Yi-Fan Lu[9], Maura R. Z. Madou[13], Anthony G. Marson[14], Heather C. Mefford[15], Sahar Esmaeeli Nieh[16], Terence J. O'Brien[17], Ruth Ottman[18], Slavé Petrovski[2,9,17], Annapurna Poduri[19], Elizabeth K. Ruzzo[9], Ingrid E. Scheffer[20,21], Elliott H. Sherr[22] & Christopher J. Yuskaitis[23]

Epilepsy Phenome/Genome Project

Bassel Abou-Khalil[24], Brian K. Alldredge[25], Jocelyn F. Bautista[26], Samuel F. Berkovic[2], Alex Boro[27], Gregory D. Cascino[28], Damian Consalvo[29], Patricia Crumrine[30], Orrin Devinsky[31], Dennis Dlugos[5], Michael P. Epstein[7], Miguel Fiol[32], Nathan B. Fountain[33], Jacqueline French[12], Daniel Friedman[12], Eric B. Geller[34], Tracy Glauser[8], Simon Glynn[35], Sheryl R. Haut[36], Jean Hayward[37], Sandra L. Helmers[38], Sucheta Joshi[39], Andres Kanner[40], Heidi E. Kirsch[13,41], Robert C. Knowlton[42], Eric H. Kossoff[43], Rachel Kuperman[44], Ruben Kuzniecky[12], Daniel H. Lowenstein[13], Shannon M. McGuire[45], Paul V. Motika[46], Edward J. Novotny[47], Ruth Ottman[18], Juliann M. Paolicchi[24,48], Jack M. Parent[49,50], Kristen Park[51], Annapurna Poduri[19], Ingrid E. Scheffer[20,21], Renée A. Shellhaas[52], Elliott H. Sherr[22], Jerry J. Shih[53], Rani Singh[54], Joseph Sirven[55], Michael C. Smith[40], Joseph Sullivan[13], Liu Lin Thio[56], Anu Venkat[5], Eileen P. G. Vining[57], Gretchen K. Von Allmen[58], Judith L. Weisenberg[59], Peter Widdess-Walsh[34] & Melodie R. Winawer[60]

[1]Department of Biostatistics and Bioinformatics, Duke Clinical Research Institute, and Center for Human Genome Variation, Duke University Medical Center, Durham, North Carolina 27710, USA. [2]Epilepsy Research Centre, Department of Medicine, University of Melbourne (Austin Health), Heidelberg, Victoria 3084, Australia. [3]Centre of Excellence in Neuromics and CHUM Research Center, Université de Montréal, CHUM-Hôpital Notre-Dame Montréal, Quebec H2L 4M1, Canada. [4]Department of Neurology, Beaumont Hospital and Royal College of Surgeons, Dublin 9, Ireland. [5]Department of Neurology and Pediatrics, The Children's Hospital of Philadelphia, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. [6]Department of Genome Sciences, University of Washington School of Medicine, Seattle, and Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195, USA. [7]Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia 30322, USA. [8]Division of Neurology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229, USA. [9]Center for Human Genome Variation, Duke University School of Medicine, Durham, North Carolina 27708, USA. [10]Department of Neurology,

The Royal Children's Hospital Melbourne , Parkville, 3052 Victoria, Australia. [11]Centre for Clinical Translation Division of Brain Sciences, Imperial College London, London SW7 2AZ, UK. [12]Comprehensive Epilepsy Center, Department of Neurology, NYU School of Medicine, New York, New York 10016, USA. [13]Department of Neurology, University of California, San Francisco, San Francisco, California 94143, USA. [14]Department of Molecular and Clinical Pharmacology, University of Liverpool, Clinical Sciences Centre, Lower Lane, Liverpool L9 7LJ, UK. [15]Department of Pediatrics, Division of Genetic Medicine, University of Washington, Seattle, Washington 98115, USA. [16]University of California, San Francisco, California 94143, USA. [17]Departments of Medicine and Neurology, The Royal Melbourne Hospital, Parkville, Victoria 3146, Australia. [18]Departments of Epidemiology and Neurology, and the G. H. Sergievsky Center, Columbia University; and Division of Epidemiology, New York State Psychiatric Institute, New York, New York 10032, USA. [19]Division of Epilepsy and Clinical Neurophysiology, Department of Neurology Boston Children's Hospital, Boston, Massachusetts 02115, USA. [20]Epilepsy Research Centre, Department of Medicine, University of Melbourne (Austin Health), Heidelberg, Victoria 3084, Australia. [21]Florey Institute and Department of Pediatrics, Royal Children's Hospital, University of Melbourne, Victoria 3052, Australia. [22]Departments of Neurology, Pediatrics and Institute of Human Genetics, University of California, San Francisco, San Francisco, California 94158, USA. [23]Department of Neurology, Boston Children's Hospital Harvard Medical School, Boston, Massachusetts 02115, USA. [24]Department of Neurology, Vanderbilt University Medical Center, Nashville, Tennessee 37232, USA. [25]Department of Clinical Pharmacy, UCSF School of Pharmacy, Department of Neurology, UCSF School of Medicine, San Francisco, California 94143, USA. [26]Department of Neurology, Cleveland Clinic Lerner College of Medicine & Epilepsy Center of the Cleveland Clinic Neurological Institute, Cleveland, Ohio 44195, USA. [27]Department of Neurology, Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, New York 10467, USA. [28]Divison of Epilepsy, Mayo Clinic, Rochester, Minnesota 55905, USA. [29]Epilepsy Center, Neurology Division, Ramos Mejía Hospital, Buenos Aires 1221, Argentina. [30]Medical Epilepsy Program & EEG & Child Neurology, Children's Hospital of Pittsburgh of UPMC, Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15224, USA. [31]NYU and Saint Barnabas Epilepsy Centers, NYU School of Medicine, New York, New York 10016, USA. [32]Department of Neurology, Epilepsy Care Center, University of Minnesota Medical School, Minneapolis 55414, USA. [33]FE Dreifuss Comprehensive Epilepsy Program, University of Virginia, Charlottesville, Virginia 22908, USA. [34]Division of Neurology, Saint Barnabas Medical Center, Livingston, New Jersey 07039, USA. [35]Department of Neurology, Comprehensive Epilepsy Program, University of Michigan Health System, Ann Arbor, Michigan 48109, USA. [36]Comprehensive Epilepsy Center, Montefiore Medical Center, Bronx, New York 10467, USA. [37]The Kaiser Permanente Group, Oakland, California 94618, USA. [38]Neurology and Pediatrics, Emory University School of Medicine, Atlanta, Georgia 30322, USA. [39]Pediatrics & Communicable Diseases, University of Michigan, Ann Arbor, Michigan 48109, USA. [40]Department of Neurological Sciences, Rush Epilepsy Center, Rush University Medical Center, Chicago, Illinois 60612, USA. [41]Department of Radiology, University of California, San Francisco, California 94143, USA. [42]Neurology, University of Texas Medical School, Houston, Texas 77030, USA. [43]Neurology and Pediatrics, Child Neurology, Pediatric Neurology Residency Program, Johns Hopkins Hospital, Baltimore, Maryland 21287, USA. [44]Epilepsy Program, Children's Hospital & Research Center Oakland, Oakland, California 94609, USA. [45]Clinical Neurology, Children's Hospital Epilepsy Center of New Orleans, New Orleans, Louisiana 70118, USA. [46]Comprehensive Epilepsy Center, Oregon Health and Science University, Portland, Oregon 97239, USA. [47]Departments of Neurology and Pediatrics, University of Washington School of Medicine, Seattle Children's Hospital, Seattle, Washington 98105, USA. [48]Weill Cornell Medical Center, New York, New York 10065, USA. [49]Department of Neurology and Neuroscience Graduate Program, University of Michigan Medical Center, Ann Arbor, Michigan 49108, USA. [50]Ann Arbor Veterans Administration Healthcare System, Ann Arbor, Michigan 48105, USA. [51]Departments of Neurology and Pediatrics, University of Colorado School of Medicine, Children's Hospital Colorado, Denver, Colorado 80045, USA. [52]University of Michigan, Pediatric Neurology, Ann Arbor, Michigan 48109, USA. [53]Department of Neurology, Mayo Clinic, Jacksonville, Florida 32224, USA. [54]Division of Pediatric Neurology, University of Michigan Health System, Ann Arbor, Michigan 48109, USA. [55]Department of Neurology, Mayo Clinic, Scottsdale, Arizona 85259, USA. [56]Department of Neurology, Washington University School of Medicine, St Louis, Missouri 63110, USA. [57]Department of Neurology, Johns Hopkins Hospital, Baltimore, Maryland 21287, USA. [58]Division of Child & Adolescent Neurology, Departments of Pediatrics, University of Texas Medical School, Houston, Texas 77030, USA. [59]Department of Neurology, Division of Pediatric Neurology, Washington University School of Medicine, St Louis, Missouri 63110, USA. [60]Department of Neurology and the G.H. Sergievsky Center, Columbia University, New York, New York 10032, USA.

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Induction of mouse germ-cell fate by transcription factors *in vitro*

Fumio Nakaki[1], Katsuhiko Hayashi[1,2,3], Hiroshi Ohta[1,4], Kazuki Kurimoto[1,4], Yukihiro Yabuta[1,4] & Mitinori Saitou[1,2,4,5]

The germ-cell lineage ensures the continuity of life through the generation of male and female gametes, which unite to form a totipotent zygote. We have previously demonstrated that, by using cytokines, embryonic stem cells and induced pluripotent stem cells can be induced into epiblast-like cells (EpiLCs) and then into primordial germ cell (PGC)-like cells with the capacity for both spermatogenesis and oogenesis[1,2], creating an opportunity for understanding and regulating mammalian germ-cell development in both sexes *in vitro*. Here we show that, without cytokines, simultaneous overexpression of three transcription factors, *Blimp1* (also known as *Prdm1*), *Prdm14* and *Tfap2c* (also known as *AP2γ*), directs EpiLCs, but not embryonic stem cells, swiftly and efficiently into a PGC state. Notably, *Prdm14* alone, but not *Blimp1* or *Tfap2c*, suffices for the induction of the PGC state in EpiLCs. The transcription-factor-induced PGC state, irrespective of the transcription factors used, reconstitutes key transcriptome and epigenetic reprogramming in PGCs, but bypasses a mesodermal program that accompanies PGC or PGC-like-cell specification by cytokines including bone morphogenetic protein 4. Notably, the transcription-factor-induced PGC-like cells contribute to spermatogenesis and fertile offspring. Our findings provide a new insight into the transcriptional logic for PGC specification, and create a foundation for the transcription-factor-based reconstitution and regulation of mammalian gametogenesis.

In mice, PGCs, the precursors for spermatozoa and oocytes, arise in the epiblasts in response to cytokines, including bone morphogenetic protein 4 (BMP4), from extraembryonic tissues[3]. Gene-knockout studies have identified transcription factors (TFs) essential for PGC specification[3]. However, the TFs sufficient for PGC induction and the precise mechanism of action of key TFs remain unknown. Using the *in vitro* PGC specification system[1,2], we set out to identify TFs whose forced expression may be sufficient to confer the PGC fate onto EpiLCs. We focused on the three TFs BLIMP1 (hereafter referred to as B), PRDM14 (P14) and TFAP2C (A) because they show specific expression in PGCs and are necessary for PGC specification[4–7]. We derived embryonic stem (ES) cells expressing mVenus and enhanced cyan fluorescent protein (eCFP) under the control of *Blimp1* and *stella* (also known as *Dppa3* and *PGC7*) regulatory elements, respectively (*Blimp1-mVenus* (BV) and *stella-eCFP* (SC), or BVSC)[1,2,8–10], and reverse tetracycline transactivator (rtTA) under the control of the constitutively active *Rosa26* locus[11] (BVSCR26rtTA ES cells) (Fig. 1a). During mouse development, *Blimp1* expression signifies the onset of PGC specification, whereas *stella* begins expression in the established PGCs[5,8,9]. We transfected the BVSCR26rtTA ES cells (XY karyotype) (Supplementary Fig. 1a–c) with *piggyBac* transposon-based vectors expressing *Blimp1*, *Prdm14* or *Tfap2c* under the control of tetracycline regulatory elements to isolate BVSCR26rtTA ES cells bearing transgenes for all three TFs (BP14A), or two (BP14, BA and P14A) or one (B, P14 and A) of the three TFs (Fig. 1a and Supplementary Fig. 1d, e).

We first examined the effect of simultaneous forced expression of the three TFs. We induced BP14A cells (line 3-3) into EpiLCs, and generated floating aggregates of ~2,000 EpiLCs in the absence of relevant cytokines with or without doxycycline (Dox; 1.5 $\mu g\,ml^{-1}$), a tetracycline analogue (Supplementary Fig. 2a). The aggregates without Dox did not show BVSC expression over the six-day period (Fig. 1b). By contrast, remarkably, those with Dox exhibited robust BVSC expression as early as day 2 of the Dox treatment (Fig. 1b). We confirmed that Dox induces exogenous TFs rapidly and at high levels in nearly all EpiLCs (Supplementary Fig. 2b, c). Fluorescence activated cell sorting (FACS) revealed that at day 2, more than ~80% and ~30% of the cells expressed BV and SC, respectively (Fig. 1c), and the efficiency of BVSC induction was dependent on the Dox dosage (Supplementary Fig. 2d, e). The BVSC induction by Dox was much more efficient/faster than that by the cytokines[1,2] (Supplementary Fig. 2f). The Dox-induced BVSC-positive cells showed proliferation and persisted until day 4, but decreased thereafter (Fig. 1b and Supplementary Fig. 2g). The other BP14A lines showed similar BVSC induction by Dox (Supplementary Fig. 3a, b). As in the case of the induction by cytokines[1], the Dox-induced EpiLCs adhering to the culture dish tended to detach, and the remaining cells did not activate BVSC as efficiently (data not shown). Thus, under the floating aggregate condition, the three TFs are sufficient for robust activation of the BVSC transgenes in EpiLCs.

We analysed the expression of genes relevant for PGC specification in TF (BP14A)-induced BV-positive cells (including SC-positive cells; Fig. 1c) by quantitative PCR (qPCR). Like cytokine-induced BV-positive cells, TF-induced BV-positive cells upregulated key genes for PGC specification/development (*Blimp1*, *Prdm14*, *Tfap2c*, *Nanos3*, *stella*, *Pou5f1*, *Sox2* and *Nanog*) and downregulated epigenetic modifiers (*Dnmt3a* and *Dnmt3b*)[1] (Fig. 1d). Interestingly, unlike cytokine-induced cells and PGCs *in vivo*[1,8,12,13], TF-induced cells did not show transient upregulation of mesodermal genes such as *Hoxa1*, *Hoxb1* and *T* (also known as brachyury), but continued to express them at low/undetectable levels (Fig. 1d and Supplementary Fig. 3d), indicating that the TF (BP14A)-induced BV-positive cells acquire a transcriptional program similar to PGCs, but lack a mesodermal program.

We next examined whether the forced expression of two or one of the three TFs would induce BVSC (three lines for each). We found that P14A, and to a lesser extent, BP14 and BA, and, notably, P14 alone, activated BVSC, although all at lower efficiencies than all three TFs combined (BP14A) (Supplementary Fig. 3a–c). The BA-induced aggregates (two out of three lines) looked fragile and remained small (Supplementary Fig. 3a), and B or A alone did not activate BVSC (Supplementary Fig. 3a–c). We confirmed that all the lines showed uniform induction of exogenous TF(s) after Dox treatment (Supplementary Fig. 4a–f). It should be noted that we could not isolate B cells that express exogenous *Blimp1* after Dox treatment at levels as high as those of exogenous *Prdm14* in P14 cells (Supplementary Fig. 4c–e). The two TFs (P14A, BP14, BA)- and single TF (P14)-induced BV-positive cells (including SC-positive cells (Supplementary Fig. 3c)) exhibited gene expression dynamics similar to those of BP14A-induced BV-positive cells (Fig. 1d and Supplementary Fig. 4g), suggesting that once the key regulators for

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[1]Department of Anatomy and Cell Biology, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan. [2]Center for iPS Cell Research and Application, Kyoto University, 53 Kawahara-cho, Shogoin Yoshida, Sakyo-ku, Kyoto 606-8507, Japan. [3]JST, PRESTO, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan. [4]JST, ERATO, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan. [5]Institute for Integrated Cell-Material Sciences, Kyoto University, Yoshida-Ushinomiya-cho, Sakyo-ku, Kyoto 606-8501, Japan.

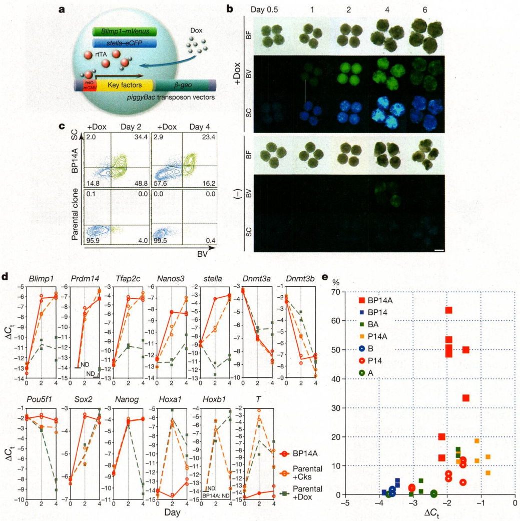

Figure 1 | Induction of a PGC-like state by TFs. **a**, Scheme for the BVSCR26rtTA cells and the *piggyBac* transposon-based vectors with tetracycline-responsive promoters driving TF and β-geo expression. **b**, Induction of BVSC with (top) or without (bottom) Dox (1.5 μg ml^{-1}) in floating aggregates of EpiLCs induced from BP14A cells during the 6-day period. BF, bright field. Scale bar, 200 μm. **c**, FACS analysis of BVSC induction by Dox (1.5 μg ml^{-1}) in floating aggregates of EpiLCs from BP14A cells (line 3-3) (top) or parental BVSCR26rtTA cells (bottom). **d**, Expression of the indicated genes measured by qPCR in TF (BP14A)- and cytokine-induced BV-positive cells (red and orange, respectively) or in the whole aggregates of parental BVSCR26rtTA EpiLCs with Dox (grey) during the 4-day period. For each gene, the ΔC_t from the average C_t values of *Arbp* and *Ppia* is shown ($\log_2$ scale, the mean value of two independent experiments with two technical replicates). Cks, cytokines; ND, not detectable. **e**, BVSC induction rates (%) at day 2 plotted against whole exogenous TF transcript levels (ΔC_t) 12 h after induction in several independent clones of the indicated cells.

PGC specification is activated, the induced cells acquire similar transcriptional profiles. We determined the relationship between the BVSC induction rate and the exogenous TF expression level. As shown in Fig. 1e (data based on Supplementary Figs 3b and 4c), BP14A induced BVSC much more efficiently than P14A or BA or P14 at similar whole exogenous TF levels, indicating that BP14A shows a synergistic effect on the activation of PGC-like transcriptional profiles in EpiLCs.

To exclude the possibility that the TFs activate BMP4 signalling, which in turn induces EpiLCs into a PGC-like state, we induced BP14A in EpiLCs with an inhibitor for BMP4 signalling—LDN193189, which inhibits activin receptor-like kinase 2/3. LDN193189 blocked BV, *Blimp1* and *Prdm14* induction by BMP4, but not by BP14A (Supplementary Fig. 5a, b). These findings, together with the much faster kinetics of BVSC induction by the TFs than by the cytokines (Fig. 1b and Supplementary Fig. 2f), indicate that the TFs directly activate a PGC program. We then examined whether induction of a PGC-like state by TFs requires an EpiLC state. Although BP14A induced EpiLCs robustly into a PGC-like state, BP14A induction in ES cells resulted in a peculiar phenotype: intense SC activation with no BV (Supplementary Fig. 5c, d), demonstrating that a proper cellular context (for example, a proper epigenetic background, presence/absence of *trans*-acting factors) is essential for the robust induction of a PGC-like state by TFs.

To characterize further the TF-induced PGC-like cells (hereafter referred to as TF-PGCLCs), we determined their transcriptomes (BV-positive cells (including SC-positive cells) induced by BP14A (days 2 and 4), BP14 (day 2), P14A (day 2) and P14 (day 2) (Supplementary Fig. 6a)) and compared them with those of PGCs at embryonic day (E) 9.5 (ref. 1) and cytokine-induced day 2, 4 and 6 PGCLCs (hereafter referred to as Ck-PGCLCs)[1]. Principal component analysis (PCA) revealed that all the TF-PGCLCs, irrespective of the TF combinations or of the induction period, bear similar transcriptomes, which are also similar to the transcriptomes of day 4 and 6 Ck-PGCLCs and, to a lesser extent, of E9.5 PGCs (Fig. 2a and Supplementary Table 1), indicating that the exogenous TFs, at varying efficiencies depending on their combinations, creates a similar PGC-like transcriptome. Notably, day 2 Ck-PGCLCs showed a more similar transcriptome to E5.75 epiblasts than to TF-PGCLCs, day 4 and 6 Ck-PGCLCs, and E9.5 PGCs, indicating that day 2 Ck-PGCLCs represent a transient state towards the acquisition of a PGC-like state from the EpiLC/epiblast states (Fig. 2a). The transcriptomes of the SC-positive cells induced by BP14A in ES cells (SC ES cells by BP14A) (Supplementary Fig. 5c, d) were different from those of PGCLCs/PGCs, and closer to those of ES cells (Fig. 2a).

We looked at individual genes upregulated in day 2 TF (BP14A)-PGCLCs in comparison to those in EpiLCs/control EpiLCs without exogenous TFs but treated with Dox, and found that genes such as *Blimp1*, *Prdm14*, *Tfap2c*, *stella*, *Sox2*, *Klf2*, *Tcl1*, *Esrrb*, *Elf3*, *Kit*, *Lifr*, *Nr5a2*, *Gjb3*, *Tdh*, *Spnb3*, *Pygl*, *Mbp*, *Npnt* and *AU015386* showed robust upregulation. All of these genes ('core PGC genes') were upregulated in day 4 and 6 Ck-PGCLCs and in E9.5 PGCs (Supplementary Fig. 6b, Supplementary Table 2). We examined the genes upregulated in day 2 Ck-PGCLCs but not in day 2 TF-PGCLCs in comparison to those in EpiLCs, which revealed that the genes *Hoxa1*, *Hoxb1*, *Hoxb2*, *Evx1*, *T*, *Cdx1*, *Cdx2*, *Hand1*, *Snai1*, *Mesp1*, *Id1*, *Msx1*, *Msx2*, *Nkx1-2*, *Isl1*, *Mixl1*, *Rspo3*, *Wnt5a*, *Fgf8* and *Bmp4*—all of which show transient upregulation in PGC precursors at around E7.0 and represent a somatic

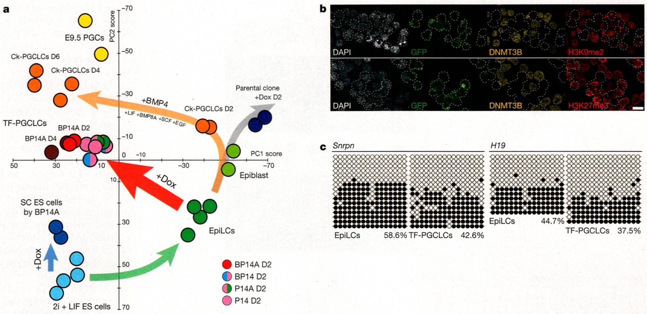

Figure 2 | Global transcription profiles for TF- and Ck-PGCLCs and epigenetic properties of TF-PGCLCs. **a**, PCA of the transcriptomes of the indicated cells. PGCLCs were sorted by BV. D, day. **b**, Immunofluorescence analysis for H3K9me2 (top) or H3K27me3 (bottom) in day 4 TF (BP14A)-PGCLCs (green fluorescent protein (GFP)-positive, dotted lines, line 3-3) compared to those in EpiLCs (DNMT3B-positive, line 3-3). Left, 4′,6-diamidino-2-phenylindole (DAPI) staining. Scale bar, 20 μm. **c**, Bisulphite sequence analysis of methylated cytosine in the differentially methylated regions of the imprinted genes in EpiLCs and day 4 BV-positive TF (BP14A, line 3-3) PGCLCs. White and black circles represent unmethylated and methylated cytosines, respectively.

mesodermal program[13]—were transiently upregulated in day 2 Ck-PGCLCs but not in day 2 TF-PGCLCs, and most of these genes were downregulated in day 4 and 6 Ck-PGCLCs and in E9.5 PGCs (Supplementary Fig. 6b and Supplementary Table 2). Genes up-/downregulated in SC ES cells by BP14A showed no correlation to those in TF-PGCLCs (Supplementary Fig. 6c and Supplementary Table 3). These findings demonstrate on a genome-wide scale that PGC/PGCLC specification by BMP4 activates both a key PGC program and a somatic mesodermal program, the latter of which is eventually repressed by the former, and that the direct activation of key TFs confers EpiLCs with the key PGC program but not the somatic mesodermal program.

We evaluated the epigenetic profiles of TF-PGCLCs. Compared to EpiLCs showing high DNMT3B, BV-positive day 4 TF-PGCLCs were extremely weak for DNMT3B and showed reduced histone H3 lysine 9 di-methylation (H3K9me2) and increased H3K27 tri-methylation (H3K27me3) (Fig. 2b and Supplementary Fig. 7). BV-positive day 4

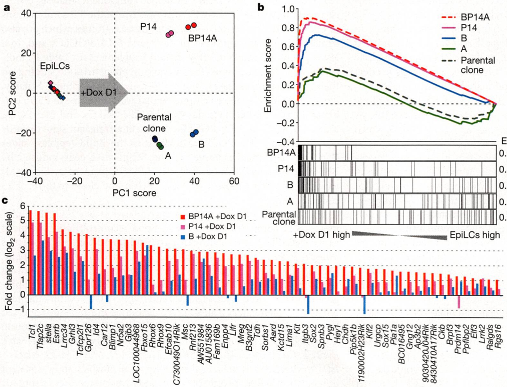

Figure 3 | Global transcriptional target analyses of the three TFs. **a**, PCA of the transcriptomes of whole EpiLC aggregates induced by the indicated factors and of corresponding parental EpiLCs. **b**, GSEA on core PGC genes of the transcriptomes of whole EpiLC aggregates induced by the indicated factors at 24 h. Genes were ranked by the difference of the expression level ($\log_2$) between day 1 aggregates and EpiLCs for each line. ES, enrichment score. **c**, Induction levels of core PGC genes in whole EpiLC aggregates by the indicated factors at 24 h. The mean values of two biological replicates for core PGC genes upregulated more than twofold in induced aggregates compared to uninduced aggregates are shown.

TF-PGCLCs retained the imprints on paternally imprinted *H19* and maternally imprinted *Snrpn* (Fig. 3c). These findings suggest that the BV-positive day 4 TF-PGCLCs acquire an epigenome similar to day 6 Ck-PGCLCs and to migrating PGCs at E8.5–E9.5 (refs 1, 14).

We went on to gain insights into the targets of the three TFs. The induction kinetics by the TFs of endogenous *Blimp1*, *Prdm14* and *Tfap2c* in whole EpiLC aggregates showed that (1) BLIMP1 and PRDM14 activate *Blimp1* gradually and *Tfap2c* rapidly, whereas TFAP2C does not activate *Blimp1* nor *Tfap2c*, but enhances the activity of BLIMP1 to induce *Tfap2c* and *Blimp1*; (2) activation of *Prdm14* by the TFs occurs relatively late (after 24 h); and (3) BP14A has the strongest effect on the activation of *Blimp1*, *Tfap2c* and subsequently *Prdm14* (Supplementary Fig. 8). We determined the transcriptomes induced by the TFs at 24 h in whole EpiLC aggregates. Unsupervised hierarchical clustering and PCA showed that PRDM14 alone induces a transcriptome similar to that induced by BP14A (Fig. 3a, Supplementary Fig. 9a and Supplementary Table 4). Accordingly, Gene Set Enrichment Analysis (GSEA)[15] showed that core PGC genes are enriched in genes induced by PRDM14 and, to a lesser extent, in genes induced by BLIMP1, but show no significant enrichment in genes induced by TFAP2C (Fig. 3b). Thus, PRDM14 has a central role in the BP14A-induced PGCLC formation.

We analysed individual genes regulated by PRDM14 and BLIMP1 with regard to the regulation of corresponding genes by BP14A. We classified the genes regulated by PRDM14 and BLIMP1 into those regulated in the same (a) (up or down by both P14 and B) and opposite (b) (up by P14 and down by B, or vice versa) direction. This revealed a trend in which the expression-level changes of the type (a) genes were enhanced (Fig. 3c and Supplementary Fig. 9b–d), whereas the type (b) genes were 'balanced' (Supplementary Fig. 9e, f) by BP14A. Importantly, the core PGC genes were overrepresented by genes upregulated by both PRDM14 and BLIMP1 (Fig. 3c). We scrutinized individual genes up-/downregulated by PRDM14 and BLIMP1. We found that PRDM14 upregulates genes associated with pluripotency (such as *Epas1*, *Tcl1*, *Esrrb*, *Klf5*, *Nr5a2*, *Zfp42*, *Klf4*, *Lifr*, *Dppa2*, *Dppa5a* and *Nanog*), and downregulates genes associated with neural differentiation (*Zfp521*, *Sox3*, *Nrcam* and *Hs6st2*). Although we did not find a clear functional category of genes upregulated by BLIMP1, we noted that BLIMP1 represses targets of OCT4 and SOX2 (*Fgf4*, *Lefty1* and *Lefty2*), among others (Supplementary Fig. 9c–f and Supplementary Table 5). Some of the targets of PRDM14 were in common between ES cells and EpiLCs (upregulated: *Tcl1*, *Esrrb* and *Klf5*; downregulated: *Hs6st2* and *Dnmt3b*)[16] (Supplementary Fig. 9c–f). These findings reveal that PRDM14 and BLIMP1 function primarily and co-operatively, whereas TFAP2C has an auxiliary role, at least for BLIMP1, for the acquisition of the TF-PGCLC transcriptome. The finding that BLIMP1 makes a relatively small contribution to the TF-PGCLC transcriptome would be attributable to TF-PGCLCs lacking the cytokine-induced somatic mesodermal program, which BLIMP1 has an essential role in repressing[13].

After Dox withdrawal, the TF-PGCLCs shut off exogenous TFs, but continue their endogenous transcription program, and may therefore serve as precursors for proper spermatogenesis. To explore this possibility, we induced TF-PGCLCs (BP14A, BP14, P14A, P14), purified the BV-positive cells (including SC-positive cells, at days 3, 4 and 6) (Fig. 4a and Supplementary Table 6), and transplanted them into seminiferous tubules of neonatal W/W^v mice lacking endogenous germ cells[1,17]. Notably, ten weeks after transplantation, the testes transplanted with the TF-PGCLCs, particularly those sorted at days 3 and 4, irrespective of the TFs used, contained numerous tubules with signs of spermatogenesis (Fig. 4b, Supplementary Fig. 10a and Supplementary Table 6). These tubules showed normal spermatogenesis and contained spermatozoa with proper morphology (Fig. 4c, d and Supplementary Fig. 10b). The control day 6 Ck-PGCLCs also contributed to spermatogenesis (Supplementary Table 6). By contrast, SC ES cells induced by BP14A did not contribute to spermatogenesis, but formed foci of teratomas in six out of eight transplanted testes (Supplementary Fig. 10d, e and Supplementary Table 6). The inability of day 6 TF-PGCLCs to contribute to spermatogenesis may result from a prolonged non-optimal culture of TF-PGCLCs (Supplementary Table 6). We fertilized wild-type oocytes with TF-PGCLC-derived spermatozoa by intracytoplasmic sperm injection[18]. The resultant zygotes developed into two-cell embryos, and we transferred these embryos into pseudopregnant female mice, and 18 days later, obtained healthy offspring with grossly normal placenta and imprinting states (Fig. 4e–h, Supplementary Fig. 10c, f and Supplementary Table 7). These offspring bore transgenes for the exogenous TFs and the BVSCR26rtTA (Supplementary Fig. 10g), and grew up normally into fertile adults (Fig. 4i, Supplementary Fig. 10h, i and Supplementary Table 7). We conclude that the TF-PGCLCs function as bona fide precursors for the spermatogenesis and healthy offspring.

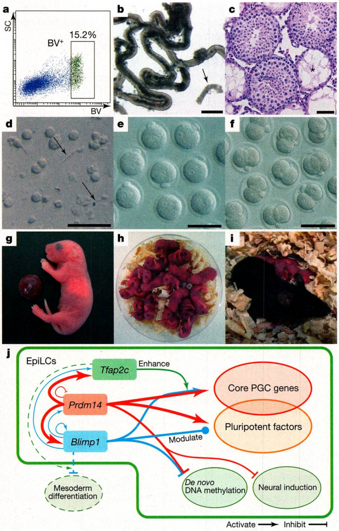

Figure 4 | Spermatogenesis and fertile offspring from TF (BP14A)-PGCLCs. a, FACS of BV-positive TF (BP14A, line 3-10)-PGCLCs for injection. **b**, Seminiferous tubules of a W/W^v mouse injected with TF-PGCLCs showing spermatogenesis. An arrow indicates an empty tubule. Scale bar, 500 μm. **c**, Haematoxylin and eosin staining of a section of a W/W^v mouse testis injected with TF-PGCLCs showing spermatogenesis. The asterisk indicates an empty tubule. **c–e**, Scale bar, 50 μm. **d**, Spermatozoa (arrows) from TF-PGCLCs. **e**, Zygotes at pronuclear stages generated by injection of TF-PGCLC-derived spermatozoa into wild-type oocytes by intracytoplasmic sperm injection. **f**, Two-cell embryos from zygotes in **e**. **g**, **h**, Apparently normal offspring (**g**, **h**) and placenta (**g**) derived from TF-PGCLC-derived spermatozoa. **i**, A fertile female derived from a TF-PGCLC-derived spermatozoon. **j**, A proposed model for TF-PGCLC induction.

We have demonstrated the transcriptional logic by which BLIMP1, PRDM14 and TFAP2C activate a key PGC program and create the TF-PGCLCs (Fig. 4j). On the basis of our findings, it should be feasible to explore TF-based regulation of further crucial processes of germ-cell development. The TF-based control of germ-cell development may be applicable to mammals other than mice, including humans.

METHODS SUMMARY

BVSCR26rtTA ES cells were established and maintained under the N2B27 '2i + LIF' condition[19]. PB-TET vectors[20] with *Avi-Blimp1*, *3×FLAG-Prdm14* and *V5-Tfap2c* (Supplementary Table 8) were transfected into the BVSCR26rtTA ES cells (XY karyotype) on feeders with Lipofectamine2000 (Invitrogen) together with pPBCAG-hph and pCAGGS-mPB plasmids. Transgene integration was determined by PCR and Southern blotting. Transfected ES cells were adapted to a feeder-free condition and differentiated into EpiLCs as reported[1]. After 36 h of differentiation, cells were aggregated (2,000 cells per aggregate) in GK15 medium[1] with Dox ($1.5\,\mu\mathrm{g\,ml}^{-1}$) (Clonetech). Ck-PGCLCs were induced by BMP4, BMP8A, SCF, LIF and EGF as described[1]. Transcriptomes were analysed by GeneChip Mouse Genome 430 2.0 Array (Affymetrix)[1,21]. Published data (Gene Expression Omnibus (GEO) accessions GSM744093–GSM744096 and GSM744099–GSM744104)[1] were included in the analysis. Seminiferous tubule injection and intracytoplasmic sperm injection were performed as described[1,17,18]. All primer sequences for PCR are listed in Supplementary Table 9.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions F.N. designed and conducted the experiments, analysed the data, and wrote the manuscript. K.H. designed the experiments and analysed the data. K.K. conducted the microarray experiments. H.O. performed the transplantation of cells into seminiferous tubules. Y.Y. analysed the data. M.S. conceived the project, designed the experiments and wrote the manuscript.

Author Information The accession number for the microarray data presented in this study is available from the Gene Expression Omnibus (GEO) database under accession GSE46855. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.S. (saitou@anat2.med.kyoto-u.ac.jp).

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The pluripotent genome in three dimensions is shaped around pluripotency factors

Elzo de Wit[1]*, Britta A. M. Bouwman[1]*, Yun Zhu[1], Petra Klous[1], Erik Splinter[1], Marjon J. A. M. Verstegen[1], Peter H. L. Krijger[1], Nicola Festuccia[2], Elphège P. Nora[3], Maaike Welling[1], Edith Heard[3], Niels Geijsen[1,4], Raymond A. Poot[5], Ian Chambers[2] & Wouter de Laat[1]

It is becoming increasingly clear that the shape of the genome importantly influences transcription regulation. Pluripotent stem cells such as embryonic stem cells were recently shown to organize their chromosomes into topological domains that are largely invariant between cell types[1,2]. Here we combine chromatin conformation capture technologies with chromatin factor binding data to demonstrate that inactive chromatin is unusually disorganized in pluripotent stem-cell nuclei. We show that gene promoters engage in contacts between topological domains in a largely tissue-independent manner, whereas enhancers have a more tissue-restricted interaction profile. Notably, genomic clusters of pluripotency factor binding sites find each other very efficiently, in a manner that is strictly pluripotent-stem-cell-specific, dependent on the presence of Oct4 and Nanog protein and inducible after artificial recruitment of Nanog to a selected chromosomal site. We conclude that pluripotent stem cells have a unique higher-order genome structure shaped by pluripotency factors. We speculate that this interactome enhances the robustness of the pluripotent state.

In recent years, several technological advances have made it possible to delineate the three-dimensional shape of the genome[3]. Spatial organization of DNA has been recognized as an additional regulatory layer of chromatin, important for gene regulation and transcriptional competence[4,5]. In somatic cells active and inactive chromosomal regions are spatially segregated[6,7]. Recently, the genome was further shown to be subdivided into evolutionarily conserved topological domains[1,2].

4C (chromosome conformation capture combined with sequencing) is a genome-scale variant of the 3C technology[8], which examines the spatial organization of DNA and measures the contact frequencies of a chosen genomic site, or 'viewpoint', with the rest of the genome. To assess chromosome topology in mouse E14 embryonic stem cells (IB10), we generated high-resolution contact maps using 4C sequencing[9] for a series of individual sites representative of different chromosomal regions on various chromosomes (Supplementary Fig. 1 and Methods). All 4C experiments show the typical result of a chromosome conformation capture experiment, with the bulk of the signal close to the viewpoint, intrachromosomal captures outnumbering interchromosomal captures, and clustering of captures at distal sites[6,7] (Supplementary Fig. 2 and Supplementary Table 1). To identify significant intra- and interchromosomal contacts, we used a windowing approach in combination with a false discovery rate (FDR) analysis that determines significant clustering of independently captured sequences (FDR, $\alpha = 0.01$; ref. 10). Contacts in this case can mean either direct interactions between the chromatin of chromosomal regions or indirect contacts via intermediate protein complexes. 3D-DNA fluorescence *in situ* hybridization (FISH) experiments validated the 4C results (Supplementary Fig. 3).

Different from what is observed in somatic cells, in embryonic stem cells we find that transcriptionally inactive regions form low numbers of specific long-range contacts (Fig. 1a, b). This is not due to their inability to reach over large distances, but instead to a more random organization of their long-range captures (Supplementary Fig. 4), suggesting that inactive chromatin is spatially less organized in pluripotent nuclei. We confirmed these results in an independent, 129/Cast, embryonic stem (ES) cell line[11] (Supplementary Fig. 5a). We furthermore show that this is not an intrinsic feature of the selected regions as they do engage in many long-range contacts in astrocytes (Fig. 1c and Supplementary Fig. 6). For example, the chemoreceptor *Tas2r110* gene, part of a cluster of taste receptors that is specifically expressed in taste buds, engages in only three contacts in ES cells but shows 34 specific contacts in astrocytes (Fig. 1d).

We assessed whether the lack of long-range contacts is a global feature of ES cells, by analysing a recently published mouse ES cell Hi-C data set[1]. 'Virtual 4C' contact profiles extracted from the Hi-C data set (see Methods for details) correlate strongly to our 'true 4C' profiles (Supplementary Fig. 7), emphasizing the high level of agreement between the data sets. The Hi-C data confirm on a global scale that inactive and active chromatin differ in their propensity to form specific long-range contacts in ES cells. Similar to our 4C data, this difference is abolished in differentiated cells (cortex), where both active and inactive chromatin engage equally well in specific long-range contacts (Fig. 1e, f and Supplementary Fig. 8).

We next asked whether chromosomal organization is reversed during cellular reprogramming. 129/Cast neural precursor cells (NPCs)[9] were transduced with a lentivirus containing a multicistronic transcript encoding *Oct4* (also known as *Pou5f1*), *Klf4*, *Sox2* and *c-Myc*, to generate induced pluripotent stem (iPS) cells. Quantitative PCR (qPCR) expression analysis of several marker genes confirmed reprogramming (Supplementary Fig. 9). A reactivated gene (*Nanog*) gains contacts during iPS cell reprogramming, whereas a resilenced gene (*Ptprz1*) loses all but two contacts (Supplementary Fig. 10), demonstrating that cellular reprogramming is accompanied by the reemergence of a pluripotency-specific spatial organization of the genome.

A closer inspection of the intrachromosomal contacts made by the *Nanog* gene revealed another aspect of the 3D pluripotent genome; *Nanog* was found to interact with many genes that are known to have an important role in maintenance of ES cell pluripotency, including *Rybp*, *Ezh2*, *Tcf3* and *Smarcad1*. The ES-cell-specific nature of these contacts becomes obvious from a comparison between the ES cell and NPC contact profiles of the *Nanog* gene. Most of the ES-cell-specific interacting regions have a high density of binding sites for the pluripotency factors Oct4, Sox2 and Nanog (Fig. 2a, b). Importantly, we also find such preferential associations when we apply 4C to the *Sox2* enhancer (Supplementary Fig. 11). The ES cell Hi-C data[1] show that

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[1] Hubrecht Institute-KNAW & University Medical Center Utrecht, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. [2] MRC Centre for Regenerative Medicine, Institute for Stem Cell Research, School of Biological Sciences, University of Edinburgh, 5 Little France Drive, Edinburgh EH16 4UU, UK. [3] Mammalian Developmental Epigenetics Group, Institut Curie CNRS UMR3215 INSERM U934, Paris, France. [4] Utrecht University, School of Veterinary Medicine, 3584 CL Utrecht, The Netherlands. [5] Department of Cell Biology, Erasmus Medical Center, Rotterdam 3015 GE, The Netherlands.

*These authors contributed equally to this work.

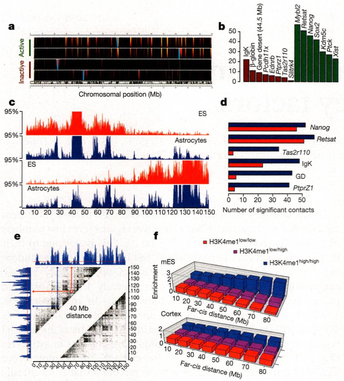

Figure 1 | Inactive regions lack specific long-range interactions in embryonic stem cells. **a**, Domainogram analysis (see Methods) shows 4C profiles of *Retsat*, *Nanog*, *Ptprz1* and gene desert (top to bottom) in ES cells. Plots represent contact profiles of active (dark green; $n = 7$) and inactive (dark red; $n = 8$) viewpoints. Below the domainograms, a map of the chromosomal position of the genes is plotted. **b**, Quantification of the number of significantly contacted regions for different viewpoints in ES cells. Green bars denote viewpoints in active regions, red bars denote viewpoints in inactive regions. **c**, Chromosomal maps show read count distribution for a gene desert (at 44.5 Mb) and for *Tas2r110*, for ES cells (red) and astrocytes (blue). The 4C signal is calculated using a sliding window average (running mean) of the read counts (window size is 51). The vertical axis is maximized at the ninety-fifth percentile. **d**, Quantification of the number of far-*cis* regions that are significantly contacted by a given viewpoint in ES cells (red) or astrocytes (blue). GD, gene desert. **e**, A pairwise contact matrix was generated to calculate disorganization scores from the Hi-C data (see Methods). Chromosome 6 was segmented into regions with high density of H3K4me1 and low density of H3K4me1, as a proxy for active and inactive chromatin. The pairwise contact matrix was subdivided into contacts between two regions of high H3K4me1 density (H3K4me1$^{\mathrm{high/high}}$) or low H3K4me1 density (H3K4me1$^{\mathrm{low/low}}$) or between a region with low H3K4me1 density and a region with high H3K4me1 density (H3K4me1$^{\mathrm{low/high}}$). **f**, From the distribution of H3K4me1 high and low regions, we calculated an expected distribution of long-range contacts, under the null hypothesis that there is no difference between active and inactive regions with respect to their long-range contacts. An enrichment score is calculated by dividing the observed scores by these expected values.

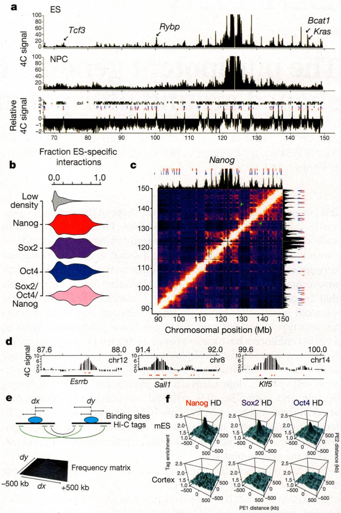

Figure 2 | Expressed *Nanog* gene shows preferential interaction with other pluripotency genes. **a**, Chromosomal map of 4C signal for the *Nanog* gene in ES cells and NPCs. Representative 4C data ($n = 6$ (ES cell) and $n = 2$ (NPC) biological replicates) is normalized to reads per million and plotted as a running mean with a window of 31. Bottom panel shows the ES cell to NPC ratio. Red, purple and blue rectangles denote the windows with a high density of Nanog-, Sox2- and Oct4-binding sites, respectively. High-density is defined as >5 sites per 100 kb. **b**, Violin plots show quantification of ES cell/NPC ratios for regions with a high density of binding sites for Nanog, Sox2, Oct4 and all three combined. **c**, Combined Hi-C–4C plot for the telomeric region of chromosome 6, shows a normalized Hi-C contact matrix (see Methods) with the 4C data for *Nanog* superimposed. Red, purple and blue rectangles show the high-density regions, as in **a**. Green arrowheads point to Nanog high-density (HD) Hi-C interactions other than with the *Nanog* enhancer. **d**, Examples of interchromosomal contacts made by *Nanog* with pluripotency genes *Esrrb*[27], *Sall1* (ref. 28) and *Klf5* (ref. 29). See methods for the definition of the 4C enrichment score. Nanog-, Sox2- and Oct4-binding sites are again highlighted with red, purple and blue rectangles. **e**, Schematic depiction of paired-end spatial chromatin analysis (PE-SCAn). Hi-C di-tags are sequentially aligned to ChIP-seq binding sites. From the total set of distances *dx* and *dy*, a normalized two-dimensional frequency matrix is calculated (see Methods). Hi-C pairs within 5 Mb were excluded to focus the analysis on contacts between, rather than within topological domains. **f**, PE-SCAn plots show the alignment of intrachromosomal Hi-C data to high-density clusters of pluripotency factors (≥5 sites in 50 kb, Nanog: $n = 423$, Sox2: $n = 607$, Oct4: $n = 1025$). Top row shows alignment of ES cell Hi-C data, bottom row shows alignment of cortex Hi-C data.

Nanog-contacting regions also form preferential contacts among each other (Fig. 2c). Among the interchromosomal contacts made by the *Nanog* gene are again a large number of pluripotency related genes: *Mybl2*, *Dppa5*, *Rex1* (also known as *Zfp42*), *Zfp281*, *Lefty1*, *Lin28a*, *Esrrb*, *Klf5*, *Sall1*, *Cbx5* and *Cbx7* (Fig. 2d and Supplementary Fig. 12; see Supplementary Table 2 for a full list of contacted regions). The contacts of *Nanog* with *Esrrb* and *Zfp281* were verified by 3D-DNA FISH (Supplementary Fig. 12). GREAT analysis[12] of these interchromosomal contacts reveals strong enrichment for genes involved in pluripotency and early embryogenesis, which is not observed for unrelated viewpoints or in other tissues (Supplementary Table 3). This suggests that pluripotency genes prefer to cluster with other pluripotency-specific genes.

We designed a computational strategy, paired-end spatial chromatin analysis (PE-SCAn) (Fig. 2e), which combines chromatin factor binding data and Hi-C data to analyse, on a global scale, whether given genomic sites (bound by a protein of interest) in different topological domains have a preference to interact among each other. PE-SCAn shows that individual Nanog-, Sox2- or Oct4-binding sites have little preference to contact each other over such large chromosomal distances (Supplementary Fig. 13a). However, clusters of Nanog-, Oct4- or Sox- binding sites (5 or more per 50 kilobases (kb)) do show a strong preference to interact with each other in ES cells (Fig. 2f). When we circularly permute the positions of the Nanog, Sox2 or Oct4 clusters, this preference is not observed, confirming that these interactions are specific (data not shown). Moreover, these contacts are tissue-specific as they are absent in the cortex (Fig. 2f).

We also used PE-SCAn to investigate the contribution of other factors to the shape of the pluripotent genome. Although CTCF and cohesin have both been implicated in higher-order chromatin folding[13], CTCF has been suggested to predominantly form chromatin loops over shorter distances[14,15]. Indeed, we find that CTCF, but also cohesin-binding sites, contribute little to chromosome folding over larger distances (Fig. 3a and Supplementary Fig. 13b). Recent chromosome architecture experiments have revealed a central role for promoters in chromosome topology[16]. PE-SCAn for histone H3 trimethyl Lys 4 (H3K4me3) confirmed that active transcriptional start sites are engaged in specific long-range contacts (Fig. 3b). However, their contribution is largely tissue-invariant, because promoters marked by H3K4me3 in either ES cells or cortex also find each other equally well in the corresponding tissue (Fig. 3b). This is different for active enhancer sites (H3K27ac[17]), which contribute to genome topology in a more tissue-restricted manner (Fig. 3b). Pluripotency factors, but also cohesin, often bind to enhancer sequences. For Oct4, Sox2, Nanog and cohesin we find that 41%, 38%, 35% and 27%, respectively, of binding sites overlap with active enhancer sites. All intersected enhancer sites show an equal preference for homotypic contacts as the unselected enhancers (Fig. 3c). Importantly, the preferred contacts among *Nanog* enhancers were not dependent on cohesin (Fig. 3d). Finally, we assessed chromosomal contacts among, respectively, enhancer and cohesin clusters (5 or more per 50 kb). We found that they have no advantage over isolated sites to interact with each other, and that their contact preference is not as pronounced as seen for clusters of pluripotency-factor-binding sites (Supplementary Fig. 13b).

To investigate whether this pluripotency-specific genome configuration is dependent on pluripotency factors, we used ZHBTc4 (ref. 18) and RCNβH (ref. 19) ES cell lines, which allow the acute depletion of Oct4 and Nanog protein, respectively (Fig. 4a, b and Supplementary Fig. 14a, b). After Oct4 or Nanog protein removal, the overall chromosome topology is largely unaffected (Fig. 4c and Supplementary Fig. 14c). However, a close comparison between factor-depleted and wild-type contact profiles reveals a decrease in contact frequencies specifically at clusters where pluripotency factors normally bind (Fig. 4d, e). Quantification confirms that the regions with reduced contact frequency after removal of Oct4 or Nanog protein are those with a high density of cognate binding sites and not, for example, regions with a high density of CTCF-binding sites (Fig. 4f, see Methods for details). Of note, partial loss of Nanog by short interfering RNA (siRNA)-mediated knockdown (78%) has no effect (Supplementary Fig. 15), indicating that full knockout of Nanog protein is required to affect chromosome topology in ES cells.

To test further whether pluripotency factor binding has a direct role in this pluripotent-stem-cell-specific genome configuration, we made use of a C56Bl/6–129S1/SvImJ ES cell line with a 256× lacO repeat cassette integrated into the C56Bl/6 *Nfix* allele on chromosome 8 (Fig. 4g and Supplementary Fig. 16). We targeted green fluorescent protein (GFP)–LacR–Nanog fusion proteins to these lacO repeats and performed allele-specific 4C (ref. 20) to simultaneously analyse the contact spectra of the targeted C56Bl/6 and the non-targeted 129S1/SvImJ allele. Again, the overall chromosome topology for both alleles was highly similar, but several new specific contacts were found for the C56Bl/6 allele. Notably, these contacts coincide with high-density Nanog-binding sites around the pluripotency genes *Sall1* and *Klf2* and the *Irx* cluster of developmental regulators. Circular permutation of the positions of the high-density Nanog clusters showed that increased contact frequency was significantly enriched at these sites ($P < 0.001$), demonstrating that Nanog has a direct role in bringing together distantly located clusters of Nanog-binding sites.

Our data show that pluripotency transcription factors shape the pluripotent genome via spatial intra- and interchromosomal gathering of high-density binding sites. It has been suggested previously that transcription factors position tissue-specific and co-regulated genes in somatic cells[21–23]. However, in contrast to previous studies, we validated this concept by comparing genome-wide contact maps

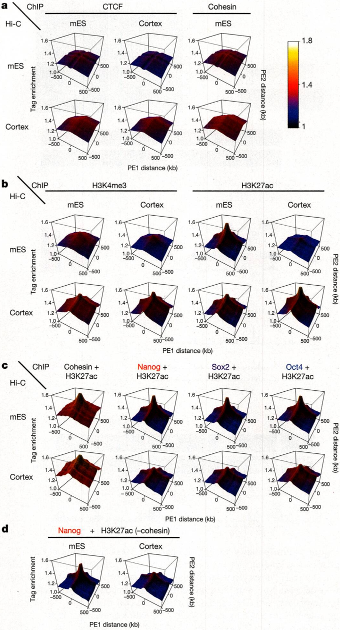

Figure 3 | Spatial interactome of chromatin factors is revealed by PE-SCAn. a–d, PE-SCAn plots for various chromatin factors. Plots are the same as in Fig. 2e, but with a different scale on the vertical axis. Note that the height of the data is colour-coded according to the colour bar shown in **a**. Top row represents mouse ES cell Hi-C data, bottom row represents cortex Hi-C data. **a**, PE-SCAn plots for known looping factors CTCF and cohesin (Smc1). **b**, PE-SCAn plots for promoter (H3K4me3) and active enhancer (H3K27ac) marks in mouse ES cells and cortex. **c**, PE-SCAn plots for active enhancer sites co-bound by cohesin (Smc1), Nanog, Sox2 or Oct4. **d**, PE-SCAn plots for genomic sites with active enhancer marks and Nanog binding, but which are devoid of cohesin. Left, mouse ES cell Hi-C data; right, cortex Hi-C data.

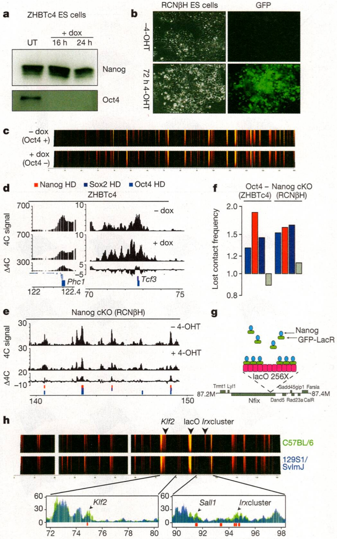

Figure 4 | Pluripotency factors influence the 3D organization of the genome. **a**, Immunoblot analysis before and after treatment of ZHBTc4 cells with 1 μg ml^{-1} doxycycline for the indicated times. Oct4 and Nanog proteins were detected using anti-Oct4 and anti-Nanog antibodies. UT, untreated. **b**, Morphology and GFP expression of RCNβH cells before and 72 h after treatment with 1 μM tamoxifen (4-OHT, 4-hydroxytamoxifen). **c**, 4C domainograms for Oct4-positive (−dox) and Oct4-negative (+dox) ZHBTc4 cells ($n = 2$ biological replicates) show that overall chromosome topology is maintained in Oct4-depleted cells. **d**, Zoomed-in regions show 4C signal (reads per million) for Oct4-positive (top) and Oct4-negative (middle) cells. Bottom, the difference (Δ) between the 4C signal of the Oct4-positive and -negative cells. Red, purple and blue rectangles show high-density Nanog, Sox2 and Oct4 regions, respectively. **e**, Same as **d** but for Nanog conditional knockout (cKO) ($n = 1$). Note that gene information is left out at this scale. **f**, Chromosome-wide analysis of differential 4C interactions for the *Nanog* enhancer viewpoint. Loss of 4C contact frequency is defined as a lower 4C signal in the knockout compared to the non-depleted reference cell line. Loss of contact frequency is determined for high-density Oct4, Nanog, Sox2 and CTCF (control) clusters for the Oct4-ablated and Nanog-conditional knockout cell lines and the enrichment over the background is calculated (see Methods for details). **g**, Schematic drawing depicting the integration site of the lacO repeat cassette in the C57Bl/6 allele of the *Nfix* locus and the targeting of GFP –LacR–Nanog fusion proteins. **h**, Domainograms showing allele-specific 4C (ref. 20) for the C56Bl/6 (containing the lacO cassette) and the 129 allele present in the hybrid ES cells ($n = 1$). Bottom panels show zoomed-in 4C profiles (C57Bl/6 green, 129 blue) for example differential regions. Red rectangles indicate high-density Nanog clusters (6 sites per 100 kb).

generated in wild-type and transcription factor knockout cells and by studying an artificially induced cluster of binding sites. Our observation that targeting or removing a given factor to or from the genome only changes specific contacts while the overall folding of chromosomes remains intact is in accordance with a recently proposed model for chromosome topology. This 'dog-on-a-lead' model predicts that chromosomes are dominant over their individual segments (genes, domains, enhancers) in dictating the overall shape of the genome, but that segments can search the nuclear subvolumes they occupy for preferred contact partners[24]. There is accumulating evidence that stochastically determined nuclear environments can influence the transcriptional output of resident genes, leading to cell-to-cell variability[25,26]. We propose that the observed spatial clustering of pluripotency factor binding sites in pluripotent stem cells enhances the transcription efficiency of nearby genes and thereby contributes to the robustness of the pluripotent state.

METHODS SUMMARY

4C sequencing and mapping. 4C sequencing was performed as previously described[9]. We used HindIII as the first restriction enzyme to generate the 3C template, which was further trimmed with DpnII. Sequencing was performed on Illumina GAII and HiSeq 2000 over multiple runs. Raw sequencing data and mapped wig files can be found under Gene Expression Omnibus (GEO) accession GSE37275.
PE-SCAn. To assess which factors are associated with genome organization, we aligned ChIP data to the Hi-C data. For this, intrachromosomal captures that are >5 Mb from each other are aligned to transcription-factor -binding sites. Only captures where both di-tags mapped within 500 kb of a ChIP peak were considered in the analysis. As a result we get for every pair of ChIP peaks on the same chromosome a set of two distances (dx, dy), to all the Hi-C di-tags that are found within 500 kb of these peaks. From the distribution of dx and dy a frequency matrix is calculated with a bin size of 50 kb, which is normalized by dividing by a randomized data set that is calculated by aligning the Hi-C data to a circularly permuted ChIP-seq data set, that is, the ChIP peaks are linearly shifted 10 Mb along the chromosome. In this manner the structure of the Hi-C data is preserved; the structure of the ChIP data is also preserved, only shifted.
Depletion of pluripotency factors. RCNβH cells were treated with tamoxifen and replated the next day. Seventy-two hours after initial tamoxifen treatment, cells were collected for 4C template preparation and analyses. ZHBTc4 cells were collected after 48 h of treatment with 1 μg ml^{-1} doxycycline.

Full Methods and any associated references are available in the online version of the paper.

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Author Contributions E.d.W. conceived the study, analysed the data and wrote the manuscript. B.A.M.B. designed and performed reprogramming and knockout experiments, and helped to write the manuscript. Y.Z. and P.H.L.K. designed and performed LacR–Nanog experiments. E.S. and P.H.L.K. performed cell culture and 4C experiments. M.J.A.M.V., E.P.N. and E.H. designed, performed and analysed FISH experiments. M.W. and N.G. assisted with reprogramming experiments. R.A.P. shared Oct4 conditional knockout cells and assisted with depletion experiments. N.F. and I.C. shared conditional knockout cells and assisted with Nanog depletion experiments. W.d.L. conceived the study and wrote the manuscript

Author Information 4C sequencing data and mapped wig files have been submitted to the Gene Expression Omnibus (GEO) under accession number GSE37275. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to W.d.L. (w.delaat@hubrecht.eu).

LETTER

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Mechanism of MEK inhibition determines efficacy in mutant KRAS- versus BRAF-driven cancers

Georgia Hatzivassiliou[1], Jacob R. Haling[2], Huifen Chen[3], Kyung Song[1], Steve Price[4], Robert Heald[4], Joanne F. M. Hewitt[4]†, Mark Zak[3], Ariana Peck[2]†, Christine Orr[1], Mark Merchant[1], Klaus P. Hoeflich[1], Jocelyn Chan[1], Shiuh-Ming Luoh[1], Daniel J. Anderson[5]†, Mary J. C. Ludlam[5]†, Christian Wiesmann[6]†, Mark Ultsch[6], Lori S. Friedman[1], Shiva Malek[2] & Marcia Belvin[1]

KRAS and BRAF activating mutations drive tumorigenesis through constitutive activation of the MAPK pathway. As these tumours represent an area of high unmet medical need, multiple allosteric MEK inhibitors, which inhibit MAPK signalling in both genotypes, are being tested in clinical trials. Impressive single-agent activity in BRAF-mutant melanoma has been observed; however, efficacy has been far less robust in KRAS-mutant disease[1]. Here we show that, owing to distinct mechanisms regulating MEK activation in KRAS- versus BRAF-driven tumours[2,3], different mechanisms of inhibition are required for optimal antitumour activity in each genotype. Structural and functional analysis illustrates that MEK inhibitors with superior efficacy in KRAS-driven tumours (GDC-0623 and G-573, the former currently in phase I clinical trials) form a strong hydrogen-bond interaction with S212 in MEK that is critical for blocking MEK feedback phosphorylation by wild-type RAF. Conversely, potent inhibition of active, phosphorylated MEK is required for strong inhibition of the MAPK pathway in BRAF-mutant tumours, resulting in superior efficacy in this genotype with GDC-0973 (also known as cobimetinib), a MEK inhibitor currently in phase III clinical trials. Our study highlights that differences in the activation state of MEK in KRAS-mutant tumours versus BRAF-mutant tumours can be exploited through the design of inhibitors that uniquely target these distinct activation states of MEK. These inhibitors are currently being evaluated in clinical trials to determine whether improvements in therapeutic index within KRAS versus BRAF preclinical models translate to improved clinical responses in patients.

The MAPK/ERK kinase (MEK) signalling cascade is a key regulator of cellular proliferation, differentiation and survival downstream of RAS activation[4]. Upregulation of this pathway occurs in a large fraction of tumours, frequently owing to oncogenic activating mutations in KRAS, NRAS, HRAS and BRAF[5]. Whereas BRAF inhibitors have shown remarkable efficacy against melanomas with BRAF(V600E) mutations, these compounds are not effective against KRAS-mutant tumours owing to inhibitor-mediated priming of wild-type RAF signalling[6–8]. As MEK is a common effector downstream of wild-type and mutant RAF[9,10], MEK inhibitors have the potential to target all tumours dependent on MAPK pathway signalling. Hence, MEK inhibitors show efficacy against both BRAF- and KRAS-mutant cancer cell lines and xenograft models[11]. Although the sensitivity of BRAF-mutant models is typically greater than that of KRAS-mutant models[9,12], the relative potency between the two genotypes varies for different inhibitors[13–15]. This difference is evident when comparing three allosteric MEK inhibitors in tumour models that have mutations in either BRAF or KRAS (Table 1 and Fig. 1). All three inhibitors are potent, ATP-uncompetitive inhibitors of MEK1 but show distinct shifts in cellular activity, with GDC-0973 having 100-fold weaker potency against a KRAS-mutant versus BRAF-mutant cell line, whereas GDC-0623 and G-573 show only 6- and 15-fold half-maximum effective concentration (EC_{50}) decreases, respectively (Table 1 and Supplementary Fig. 1). We profiled the MEK inhibitors across a panel of BRAF and KRAS-mutant cancer cell lines (Fig. 1a and Supplementary Table 1) and consistently found that GDC-0973 was less potent in KRAS versus BRAF-mutant cell lines, whereas GDC-0623 and G-573 had similar efficacy in the two genotypes. We tested clinically relevant doses of GDC-0973 and GDC-0623: the average plasma concentration of GDC-0973 over 24 h at its maximum tolerated dose (MTD) is 0.37 μM and for GDC-0623 is 0.63 μM (Musib *et al.*, manuscripts in preparation).

To test whether these differences translate to *in vivo* tumour models, we compared GDC-0973, GDC-0623 and G-573 in three KRAS-mutant xenograft models (Fig. 1b and Supplementary Fig. 2). Compounds were evaluated at their MTDs, and superior antitumour activity was observed with GDC-0623 and G-573 compared to GDC-0973 in all three KRAS models.

Treatment of KRAS-mutant cells with MEK inhibitors is expected to lead to an increase in phosphorylated MEK (pMEK) through feedback-mediated RAF activation[15,16]. We observed the expected increase in pMEK in response to GDC-0973 (Fig. 2a, top panel), but not GDC-0623 or G-573. This block of pMEK accumulation resulted in more effective inhibition of pERK by GDC-0623 and G-573. Release of negative feedback mediators occurred normally in response to all inhibitors (Supplementary Fig. 3). In a BRAF(V600E) cell line, where wild-type RAF activity is low[16], all inhibitors reduced pMEK levels (Fig. 2a lower panel)[13]. Knockdown experiments revealed that BRAF and CRAF, but not ARAF, were responsible for pMEK induction (Supplementary Fig. 4), in agreement with BRAF–CRAF heterodimers being the major mediators of signalling through activated KRAS[4].

Given that GDC-0623 and G-573 prevent MEK phosphorylation in cells, we tested whether these compounds directly inhibit RAF activity *in vitro*. Only GDC-0623 and G-573 were able to prevent MEK phosphorylation by CRAF *in vitro* (Fig. 2b, upper panel), whereas all three inhibitors were able to block MEK phosphorylation by BRAF(V600E) (Fig. 2b, lower panel). The inhibitors did not block phosphorylation of MEK(K97R) (Supplementary Fig. 5a), which is kinase dead and does not effectively bind the inhibitors (Supplementary Figs 5b and 10). These studies confirm that the MEK inhibitors do not block RAF activation directly, but rather through their effects on MEK. Furthermore, none of the inhibitors had off-target effects against other kinases (Supplementary Table 3).

Because the MEK inhibitors affect RAF activity, we proposed that they might affect RAF–MEK protein interactions. *In vitro* dimerization assays revealed that GDC-0623 and G-573 stabilize the RAF–MEK

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[1]Department of Translational Oncology, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, USA. [2]Department of Biochemical and Cellular Pharmacology, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, USA. [3]Department of Discovery Chemistry, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, USA. [4]Argenta, 8/9 Spire Green Centre, Flex Meadow, Harlow, Essex CM19 5TR, UK. [5]Department of Discovery Oncology, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, USA. [6]Department of Structural Biology, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, USA. †Present addresses: WestCHEM, School of Chemistry, University of Glasgow, Joseph Black Building, University Avenue, Glasgow G12 8QQ, UK (J.F.M.H.); MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 0XY, UK (A.P.); Novartis Institutes for Biomedical Research, Fabrikstrasse 16, CH-4002 Basel, Switzerland (C.W.); Cairn Biosciences Inc., 3534 24th Street, San Francisco, California 94110, USA (M.J.C.L.); Cleave Biosciences, 866 Malcolm Road suite 100, Burlingame, California 94010, USA (D.J.A.).

Table 1 | Biochemical and Cellular Potency of GDC-0973, GDC-0623, and G-573.

Compound	MEK1 (−ATP) K_i (nM)	MEK1 (+ATP) K_i (nM)	Proliferation EC_{50} (μM)		KRAS(G13D)/ BRAF(V600E) EC_{50}
			A375 (BRAF(V600E))	HCT116 (KRAS(G13D))	
GDC-0973	262.5	0.05	0.005	0.520	104
GDC-0623	217.5	0.13	0.007	0.042	6
G-573	196.3	0.30	0.011	0.167	15

complex whereas GDC-0973 does not (Fig. 2c and Supplementary Fig. 6a, b). RAF–MEK complex formation was specific to wild-type RAF and not BRAF(V600E), which does not form stable complexes in the presence of inhibitors (Fig. 2d and Supplementary Fig. 6a, b). These *in vitro* findings were confirmed in cells. In KRAS-mutant cells GDC-0623, and more markedly, G-573, induced dimerization of MEK with both BRAF and CRAF, whereas GDC-0973 reduced baseline heterodimer levels (Fig. 2e), coincident with elevated pMEK levels (Fig. 2f, top panel, and Supplementary Fig. 7a, b). Consistent with the biochemical data, G-573 did not affect the basal BRAF(V600E)–MEK complex in BRAF(V600E) homozygous cells, whereas it was able to effectively stabilize the CRAF–MEK complex (Supplementary Fig. 6c, d).

We theorized that RAF–MEK complex stabilization by GDC-0623 and G-573 may disfavour BRAF–CRAF complex formation in response to MEK inhibitors, and thus dampen feedback-induced signalling. Indeed, only GDC-0973 promoted BRAF–CRAF heterodimer formation (Fig. 2f, lower panel, and Supplementary Fig. 7b). Furthermore, whereas GDC-0973 induced CRAF translocation to the plasma membrane as previously described[15], G-573 treatment blocked it (Supplementary Fig. 7c). Thus, a subset of MEK inhibitors, in addition to blocking MEK activation by RAF, also block RAF activation through inhibition of BRAF–CRAF heterodimer formation and RAF plasma membrane translocation.

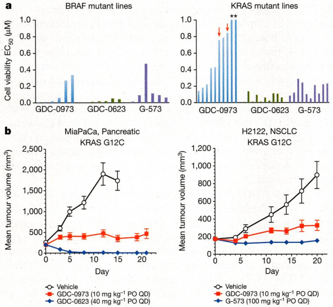

Figure 1 | Allosteric MEK inhibitors show distinct relative potencies in KRAS-mutant and BRAF-mutant cancer models. **a**, EC_{50} values (μM) in a cell viability assay of indicated MEK inhibitors against a panel of BRAF(V600E) and KRAS-mutant cancer cell lines. *EC_{50} values >1 μM. Red arrows: MiaPaCa-2 (left), H2122 (right). **b**, Antitumour efficacy of GDC-0973 in the MiaPaCa-2 and H2122 xenograft models, GDC-0623 in MiaPaCa-2 ($n = 5$ per group) or G-573 in H2122 ($n = 10$ per group). Percent tumour growth inhibition (%TGI) was 78% for GDC-0973 and 120% for GDC-0623 in MiaPaCa-2. GDC-0973 had no partial or complete regressions (PR/CR), whereas GDC-0623 had 3 PR, 2 CR. In H2122 xenografts, GDC-0973 treatment led to 80% TGI, 1 PR, 0 CR; G-573 treatment led to118% TGI, 3 PR, 0 CR. Group mean is plotted with error bars representing the standard error of mean (s.e.m.). PO QD, by mouth once a day.

We obtained a crystal structure of GDC-0973 with the MEK1 kinase domain and compared its binding mode to that of GDC-0623 and G-573 based on molecular modelling (Fig. 3a and Supplementary Fig. 10). The three inhibitors bind in a similar orientation with respect to the nucleotide and the activation loop (A-loop) helix that contains S218, one of the two MEK sites phosphorylated by RAF. S222, the second phosphorylation site, lies in a part of the activation loop that is not well defined in the

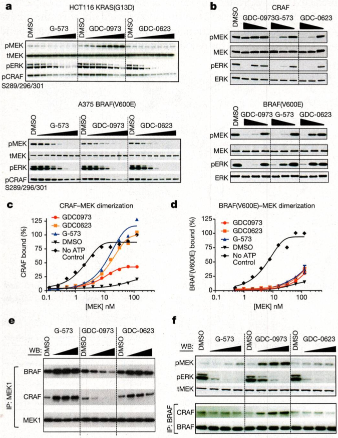

Figure 2 | MEK inhibitor efficacy in KRAS-mutant models correlates with the ability to block feedback-induced phosphorylation of MEK by RAF. **a,** Western blot (WB) of phosphorylated MEK (pMEK) and total MEK (tMEK) in HCT116 KRAS(G13D) cells (top) and A375 BRAF(V600E) cells (bottom) after overnight treatment with inhibitors (range: 0.00045–0.33 μM in threefold increments) shows pMEK induction selectively by GDC-0973 in HCT116 cells. **b,** *In vitro* cascade kinase assays using recombinant inactive MEK and ERK and recombinant kinase domains of either CRAF (top) or BRAF(V600E) (bottom) in the presence of 0.01, 0.1 and 1 μM of the indicated MEK inhibitors. **c, d,** Recombinant kinase domain (KD) $CRAF^{KD}$–MEK dimer formation (**c**) or $BRAF(V600E)^{KD}$–MEK dimer formation (**d**) are detected by homogenous time-resolved fluorescence *in vitro*, either in the absence (No ATP control) or presence of 100 μM ATP with and without MEK inhibitors (GDC-0973, GDC-0623, G-573, DMSO). **e,** Immunoprecipitation (IP)/WB of endogenous MEK1 from lysates of HCT116 (KRAS(G13D)) cells treated overnight with DMSO or indicated MEK inhibitors at 0.1, 1, 10 μM. **f,** IP/WB of endogenous BRAF from lysates of HCT116 (KRAS(G13D)) cells treated overnight with DMSO or indicated MEK inhibitors at 0.1, 1, 10 μM (lower panel). Immunoprecipitates were immunoblotted for BRAF and CRAF, showing that GDC-0973 promotes BRAF–CRAF complex formation. Total lysates were immunoblotted for pMEK, pERK and tMEK (upper panel).

crystal structure. In our structure/models, the inhibitors form an interaction with the backbone amide of S212, at the beginning of the A-loop helix[17,18]. Whereas GDC-0623 and G-573 are predicted to engage S212 with an aromatic nitrogen (N) to form a hydrogen bond, GDC-0973 engages S212 with an aromatic fluorine (F) forming a much weaker interaction, given the high electronegativity and low polarizability of F[19]. The importance of this interaction for inhibitor activity was highlighted by disrupting it through substitution of a carbon at this site, which abolished activity (Supplementary Fig. 8).

We proposed that the interaction with the S212 backbone might constrain the movement of the A-loop helix and prevent MEK phosphorylation by RAF. To test this, we mutated S212 to proline to disrupt the hydrogen bond with the inhibitors (Supplementary Fig. 9). Based on modelling, S212P also disrupts the hydrogen-bond network between the S212 side chain, the A-loop helix, and E114 in the α-C helix, and is predicted to allow greater accessibility of the A-loop to RAF (Supplementary Fig. 9). In contrast, a S212A mutation should not affect the inhibitor hydrogen-bond interaction, but would affect the hydrogen-bond network between S212, the A-loop helix and E114 and lead to increased A-loop helix dynamics and higher basal MEK activity, as previously reported[20]. In cellular studies, transfected wild-type MEK1 became highly phosphorylated in the presence of GDC-0973 (Fig. 3b). The S212P and S212A mutants showed higher basal pMEK levels, consistent with the disruption of the S212/A-loop/C-helix interactions. As predicted, GDC-0623 and G-573 were unable to inhibit the phosphorylation of MEK1(S212P) but were able to block the phosphorylation of MEK1(S212A). Our model suggests this is because of the hydrogen bonds to the alanine backbone that are lost with the proline backbone (Fig. 3b and Supplementary Fig. 9c). We propose that the stronger interaction of GDC-0623 and G-573 with S212 reduces the flexibility of the MEK A-loop helix, thus preventing RAF from accessing S218/S222.

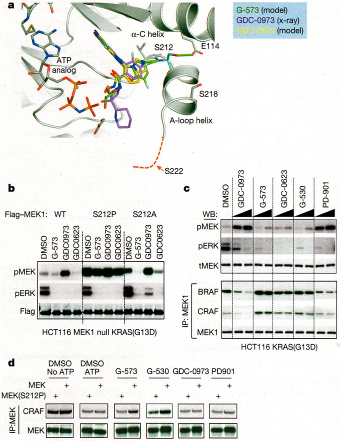

Figure 3 | Distinct inhibitor interactions with MEK determine their individual effects on RAF–MEK complex formation, MEK activation and inhibition of ERK phosphorylation. a, MEK1 crystal structure of GDC-0973 (magenta) with models of GDC-0623 (yellow) and G-573 (green) show key hydrogen-bond interactions of inhibitors with S212 on the MEK activation segment. S222 is shown in its approximate location based on modelled conformations. **b,** WB of lysates from HCT116 MEK1 null cells transiently transfected with wild-type MEK1 (WT), S212P or S212A mutant MEK1 and treated with 1 μM of the indicated MEK inhibitors for 24 h. **c,** Upper panel, WB of HCT116 (KRAS(G13D)) cells treated for 24 h with the indicated MEK inhibitors (0.1, 1 μM); lower panel, IP/WB of endogenous MEK1 from lysates of HCT116 (KRAS(G13D)) cells treated overnight with DMSO or indicated inhibitors. **d,** Recombinant protein pull-down assays using wild-type or S212P MEK after incubation with recombinant CRAF in the presence or absence of ATP (100 μM) and the indicated inhibitors.

We evaluated additional compounds with an N (G-530) or F (PD-901) at this position (Supplementary Fig. 10). As predicted by our model, G-530 prevented pMEK accumulation and stabilized a RAF–MEK complex, whereas PD-901 led to increased pMEK and lower levels of RAF–MEK dimers (Fig. 3c). In biochemical studies, RAF–MEK complexes stabilized by G-573 and G-530 were lost upon introduction of a S212P mutation in MEK (Fig. 3d). Additionally, the relative potencies of PD-901 and G-530 in KRAS versus BRAF-mutant cells track with those of GDC-0973 and GDC-0623/G-573, respectively (Supplementary Fig. 11). These data support the conclusion that a strong hydrogen bond between MEK inhibitors and S212 is important for stabilizing a RAF–MEK complex and blocking RAF-mediated MEK phosphorylation in KRAS-mutant cells.

Interestingly, the clinical MEK inhibitor AZD6244, which has an N rather than a F at the S212-interacting site, was previously reported to have lower potency in a panel of oncogenic KRAS mutant cell lines ($KRAS^{MT}$) versus BRAF(V600E) cells and to allow the induction of pMEK in $KRAS^{MT}$ cells[9,15]. Despite the presence of an N at this position, AZD6244 is predicted to form a weaker hydrogen bond with S212 compared to GDC-0623 due to a longer hydrogen-bond distance from the S212 backbone NH (Supplementary Fig. 12). This leads us to refine our model by concluding that it is the strength of the inhibitor–MEK S212 interaction, rather than the exact identity of the atom interacting with MEK that is critical for the ability of MEK inhibitors to block pMEK induction and show strong potency in KRAS-mutant cells.

Importantly, whereas GDC-0973 showed reduced efficacy in KRAS-mutant *in vivo* models compared to GDC-0623 and G-573, the reverse was found in BRAF(V600E) mutant models (Fig. 4a). In BRAF(V600E) xenografts, GDC-0973 showed the greatest efficacy at its MTD, with an increased rate of regressions. Because the rank order of compounds in efficacy studies was reversed in KRAS versus BRAF-mutant models, pharmacokinetic differences between the molecules do not account for these differences.

Given that BRAF(V600E) tumour models have high basal levels of pMEK[16] (Supplementary Fig. 13a), we proposed that GDC-0973 may have a higher binding affinity for activated pMEK, and thus greater efficacy in BRAF(V600E) cells compared to MEK inhibitors that preferentially bind to the inactive form of MEK[13,21]. To test this directly, we generated a phosphomimetic construct encoding constitutively active MEK(S218D S222D) (thereafter MEK-DD). In fluorescence polarization assays GDC-0623 and G-573 bound tenfold less strongly to MEK-DD than wild-type MEK, whereas GDC-0973 bound equivalently to both forms (Fig. 4b). We proposed that the reduction in binding affinity for GDC-0623 and G-573 against MEK-DD might be due to the weakening of their hydrogen-bond interaction with S212 as a result of increased dynamics of the A-loop and α-C helix in the MEK-DD conformation[22]. To test this, we introduced an additional mutation, E114D, to the MEK-DD protein, predicted to further increase A-loop/α-C helix dynamics and further weaken the S212 interaction with inhibitors. As predicted, GDC-0623 and G-573 lost substantial binding affinity against MEK-DD(E114D), whereas GDC-0973 was minimally affected. We attribute the strong binding of GDC-0973 to the additional interactions it makes with the nucleotide and with residues within the MEK HRD motif of the catalytic loop (Fig. 4c;

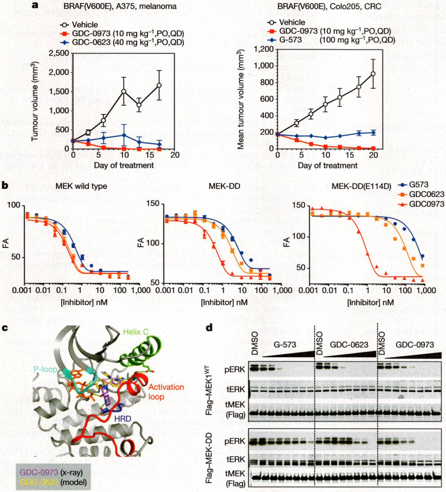

Figure 4 | Potency of MEK inhibitors in BRAF-mutant versus KRAS-mutant tumours correlates with high inhibitor binding affinity against dually phosphorylated MEK (S218/S222) compared to un-phosphorylated MEK. **a**, Antitumour efficacy of GDC-0973 in A375 and Colo205 xenograft models, GDC-0623 in A375 ($n = 5$ per group) or G-573 in Colo205 ($n = 10$ per group). In A375, GDC-0973 treatment led to 127% TGI, with 3 PR, 2 CR; GDC-0623 treatment led to 102% TGI with 3 PR, 0 CR. In Colo-205, GDC-0973 treatment led to 141% TGI with 4 PR, 6 CR; G-573 treatment led to 109% TGI with 3 PR, 0 CR. Group mean is plotted with error bars representing standard error of mean (SEM). CRC, colorectal cancer. **b**, Binding curves of GDC-0973, GDC-0623 and G-573 against MEK1^{WT}, MEK-DD and MEK-DD(E114D) using fluorescence anisotropy (FA). K_i (nM) for inhibitor are GDC-0973: 0.05, 0.18, 0.48; GDC-0623: 0.13, 1.33, ~62.0; G-573: 0.30, 3.53, ~284.0 for the MEK WT, MEKDD and MEK-DD(E114D) constructs, respectively. **c**, MEK1 crystal structure of GDC-0973 (magenta) with a model of GDC-0623 (yellow) showing GDC-0973 reaching towards the HRD motif in the catalytic loop of MEK1. **d**, WB of 293-T cells transfected with either vector, Flag–MEK1^{WT} or Flag–MEK-DD and treated for 24 h with the indicated inhibitors (range: 0.01–10 μM in threefold increments).

Supplementary Fig. 10)[23], and suggest that GDC-0973 is less affected by A-loop/α-C helix dynamics such as occurs during MEK activation.

To confirm these findings in cells, we transfected wild-type MEK or MEK-DD into cells (note: MEK-DD(E114D) is catalytically dead and thus not used) and assessed the ability of the inhibitors to prevent phosphorylation of ERK (Fig. 4d). Consistent with the binding studies, we found that GDC-0623 and G-573, but not GDC-0973, lost potency against MEK-DD.

We proposed that the strong binding of GDC-0973 to activated MEK might also explain its variable potency in KRAS-mutant cell lines. Comparison of pMEK levels in KRAS^{MT} lines that were sensitive versus resistant to GDC-0973 (Fig. 1a) demonstrated that the most sensitive lines had high basal pMEK and pERK levels comparable to those in the A375 BRAF(V600E) line (Supplementary Fig. 13). Therefore, cells with high levels of activated MEK are sensitive to GDC-0973 even in the KRAS genotype.

In conclusion, our study elucidates the mechanisms of distinct classes of allosteric MEK inhibitors that control their efficacy in KRAS versus BRAF-mutant tumours. We demonstrate that MEK inhibitors that form a strong hydrogen bond to S212 prevent phosphorylation of MEK by RAF and stabilize a RAF–MEK complex, leading to greater efficacy in KRAS-mutant tumours. Why this interaction is needed for strong inhibition of MEK activity downstream of WT RAF is not entirely understood. Other MEK inhibitors have been shown to inhibit RAF phosphorylation of MEK[13,24], although the mechanism had not previously been elucidated. Furthermore, whereas the interaction of MEK inhibitors with S212 has been previously observed[18,25], our study is the first to link directly the strength of this interaction to the prevention of RAF-mediated MEK activation and increased potency in KRAS-mutant cells.

In the context of BRAF(V600E) mutant tumours, we discovered that the superior efficacy of GDC-0973 against BRAF-mutant tumour models correlates with its potent binding and inhibition of activated MEK. Because of the high basal activity of MEK in BRAF(V600E) mutant tumours, this results in increased efficacy in this genetic background. Our data are consistent with previous studies demonstrating that MEK is activated differently by oncogenic BRAF(V600E) compared to wild-type BRAF and CRAF in tumours with oncogenic KRAS[26,27]. We demonstrate that these differences can be exploited by targeting MEK inhibitors against distinct activated states of MEK, leading to increased efficacy in BRAF or KRAS-mutant preclinical models.

With respect to the implications of these findings for the clinic, our studies were carried out with clinically relevant doses. Importantly, GDC-0973 has already shown strong clinical efficacy in BRAF(V600E) melanoma patients, with partial responses observed in 7/12 melanoma patients treated at MTD where 6 of the 7 responders had BRAF(V600E) mutations (Rosen, L. *et al.*, unpublished data)[28]. Clinical trials with GDC-0973 and GDC-0623 will assess whether the observed differences in mechanism of action translate to differential therapeutic benefit for cancer patients.

METHODS SUMMARY

GDC-0973 and G-573 were synthesized at Genentech and have been described previously[23,29]. GDC-0623 and G-530 were synthesized according to the procedure in patent number US7803839 (2010). PD0325901 (PD901) was purchased from Active Biochem. Cells were obtained from the Genentech cell line repository and were cultured according to ATCC specifications. The establishment of MiaPaCa, H2122, A375 and Colo 205 xenografts is described in detail in Methods in the Supplementary Information. Kinase reactions, immunoprecipitations and western blots were conducted as described previously[19]. The binding studies of MEK1 to small-molecule inhibitors were performed by competitive fluorescence polarization and described in Methods in the Supplementary Information. MEK1 purification and crystallization conditions are described in Methods in the Supplementary Information. Structure determination, refinement and modelling are described in Methods in the Supplementary Information and Supplementary Table 2.

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Supplementary Information is available in the online version of the paper.

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Author Information The structure of the MEK1:GDC-0973 complex has been deposited with the PDB and assigned accession code 4LMN. Reprints and permissions information is available at www.nature.com/reprints. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to to M.B. (belvin.marcia@gene.com) or G.H. (hatzivassiliou.georgia@gene.com).

LETTER

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Completion of the entire hepatitis C virus life cycle in genetically humanized mice

Marcus Dorner[1]†, Joshua A. Horwitz[1]*, Bridget M. Donovan[1]*, Rachael N. Labitt[1], William C. Budell[1], Tamar Friling[1], Alexander Vogt[1], Maria Teresa Catanese[1], Takashi Satoh[2], Taro Kawai[2], Shizuo Akira[2], Mansun Law[3], Charles M. Rice[1] & Alexander Ploss[1]†

More than 130 million people worldwide chronically infected with hepatitis C virus (HCV) are at risk of developing severe liver disease. Antiviral treatments are only partially effective against HCV infection, and a vaccine is not available. Development of more efficient therapies has been hampered by the lack of a small animal model. Building on the observation that CD81 and occludin (OCLN) comprise the minimal set of human factors required to render mouse cells permissive to HCV entry[1], we previously showed that transient expression of these two human genes is sufficient to allow viral uptake into fully immunocompetent inbred mice[2]. Here we demonstrate that transgenic mice stably expressing human CD81 and OCLN also support HCV entry, but innate and adaptive immune responses restrict HCV infection *in vivo*. Blunting antiviral immunity in genetically humanized mice infected with HCV results in measurable viraemia over several weeks. In mice lacking the essential cellular co-factor cyclophilin A (CypA), HCV RNA replication is markedly diminished, providing genetic evidence that this process is faithfully recapitulated. Using a cell-based fluorescent reporter activated by the NS3-4A protease we visualize HCV infection in single hepatocytes *in vivo*. Persistently infected mice produce *de novo* infectious particles, which can be inhibited with directly acting antiviral drug treatment, thereby providing evidence for the completion of the entire HCV life cycle in inbred mice. This genetically humanized mouse model opens new opportunities to dissect genetically HCV infection *in vivo* and provides an important preclinical platform for testing and prioritizing drug candidates and may also have utility for evaluating vaccine efficacy.

The narrow species tropism of HCV is incompletely understood. Mouse cells do not support viral entry and inefficiently replicate HCV RNA, but do support virion assembly and release. HCV uses numerous cellular factors to enter hepatocytes in a coordinated multistep process, including glycosaminoglycans[3], low-density lipoprotein receptor[4], scavenger receptor class B type I (SCARB1)[5], the tetraspanin CD81 (ref. 6), the tight junction proteins claudin 1 (CLDN1)[7] and occludin (OCLN)[1,8], the receptor tyrosine kinases epidermal growth factor receptor[9] and ephrin receptor A2 (ref. 9), and the cholesterol uptake receptor Niemann–Pick C1 like 1 (ref. 10). Of these, CD81 and OCLN comprise the minimal human factors needed for HCV uptake into rodent cells[1].

We recently demonstrated that adenoviral delivery of HCV entry factors renders mice susceptible to HCV infection[2]. This transient approach is high-throughput and allows the possibility of rapidly evaluating mutant versions of HCV entry factors. However, adenoviral gene delivery strongly induces interferon-stimulated genes, creating an environment that may antagonize HCV replication. To limit variability and to prevent vector-mediated immune activation, we generated transgenic mice stably expressing human CD81, SCARB1, CLDN1 and/or OCLN under the control of a liver-specific albumin promoter. Transgenic expression of the human orthologues of the HCV entry factors resulted in similar mRNA levels of the human and endogenous mouse genes in the murine liver (Supplementary Fig. 1) and expression of all four proteins (Supplementary Fig. 2) with the expected subcellular distribution in the liver (Supplementary Fig. 3). Next, we aimed to test the susceptibility of entry factor transgenic (EFT) mice to HCV infection. To identify founder lines supporting viral entry we took advantage of a previously generated, highly sensitive detection system which is based on the activation of a *loxP*-flanked STOP-luciferase reporter in the genome of ROSA26-Fluc mice by Cre recombinase encoded in recombinant HCV genomes[2]. We crossed EFT mice to a ROSA26-Fluc background and challenged these animals with a bicistronic HCV genome expressing Cre (HCV-Cre). Consistent with previous data[2], the bioluminescent reporter was activated in mice expressing human CD81 and OCLN (Fig. 1a and Supplementary Fig. 4a). The addition of human SCARB1 and CLDN1 (Supplementary Fig. 4b) did not increase the entry signal, demonstrating that their murine orthologues are functional for HCV entry *in vivo*. For subsequent experiments, founder lines Alb-hCD81/hOCLN#941 (2hEF) and Alb-hCD81/hSCARB1/hCLDN1/hOCLN#100 (4hEF), which supported the most efficient viral uptake (Supplementary Fig. 4a), were used. To estimate the number of HCV-infected liver cells, we used an indicator mouse strain in which Cre leads to activation of a nuclear-localized green fluorescent protein/β-galactosidase (GNZ) reporter (ROSA26-GNZ)[11]. Similar to our previous observations[2], HCV-Cre infection resulted in reporter activation in approximately 1–1.5% of murine hepatocytes in 2hEF or 4hEF mice (Fig. 1b and Supplementary Fig. 5). To provide additional evidence that viral uptake into EFT mice is mediated by the specific interaction of HCV glycoproteins with host entry factors, we administered antibodies directed against the HCV envelope glycoprotein complex E1E2 or the host entry factor CD81. Delivery of anti-human CD81 or anti-E1E2 (AR4A; ref. 12) antibodies resulted in a dose-dependent inhibition of HCV-Cre infection (Fig. 1c), whereas isotype control immunoglobulins had no effect. These data further affirm that HCV is taken up in a viral glycoprotein-specific fashion *in vivo* and underscore the utility of this model for evaluation of passive immunization strategies.

Direct measurement of HCV genome levels by quantitative reverse transcription (qRT)–PCR demonstrated a slight increase in HCV RNA in the serum (at 4 h) and liver (at 3 h and 24 h) of inoculated mice expressing the human entry factors; at 72 h, however, the signal was reduced to background levels (Fig. 1d, e). HCV infection resulted in the upregulation of several interferon-stimulated genes (Fig. 1f, g and Supplementary Fig. 6), infiltration of immune cells, especially natural killer (NK) cells, into the liver (Fig. 1i), and elevated proinflammatory cytokine levels in the serum (Fig. 1h and Supplementary Fig. 7), which could antagonize HCV replication. This hypothesis is further supported by the previous observation that HCV replicons,

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[1] Center for the Study of Hepatitis C, The Rockefeller University, New York, New York 10065, USA. [2] Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. [3] Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California 92037, USA. †Present addresses: Imperial College London, Department of Medicine, London W2 1NY, UK (M.D.); Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA (A.P.).

*These authors contributed equally to this work.

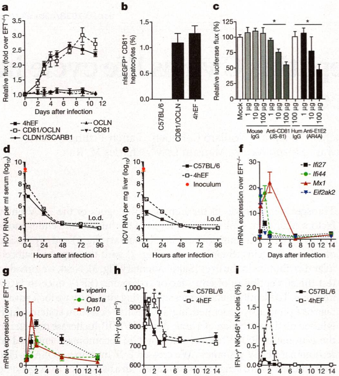

Figure 1 | Transgenic expression of human CD81 and OCLN renders mice permissive to HCV entry. **a,** Longitudinal bioluminescence imaging of ROSA26-Fluc mice expressing either human CD81 plus OCLN, SCARB1 plus CLDN1, CD81 alone, OCLN alone, or all four HCV entry factors (4hEF). **b,** Quantification of viral uptake into murine hepatocytes expressing human CD81 and OCLN or all four human entry factors determined by flow cytometry 72 h after infection with BiCre-Jc1. **c,** Blocking of HCV infection *in vivo* by either blocking antibodies against CD81 (JS-81) or neutralizing antibodies against HCV E1E2 (AR4A). ROSA26-Fluc 4hEF mice were injected with the indicated amounts of antibodies 24 h and 4 h before infection with BiCre-Jc1. **d, e,** Longitudinal quantification of HCV RNA by RT–qPCR in (**d**) serum and (**e**) liver of either wild-type or mice expressing all four HCV entry factors; dotted lines indicate the limit of detection (l.o.d.). **f, g,** Expression of the interferon-stimulated genes *Ifi27*, *Ifi44*, *Mx1* and *Eif2ak2* (**f**) and *viperin* (also known as *Rsad2*), *Oas1a* and *Ip10* (**g**) in the liver after infection with BiCre-Jc1 in wild-type or 4hEF mice. **h,** Serum levels of IFN-γ in wild-type or 4hEF mice infected with BiCre-Jc1. **i,** Analysis of liver-infiltrating IFN-γ-secreting NKp46-positive NK cells in BiCre-Jc1-infected wild-type or 4hEF mice. All data shown are mean ± s.d. of four independent experiments. For panels **e–g**, four mice were used per time point. $^*P < 0.05$.

selectable HCV RNA genomes, replicate more efficiently in murine cells with impaired antiviral signalling[13,14].

To identify a murine environment more conducive to HCV replication we crossed 4hEF ROSA26-Fluc mice to strains carrying targeted disruptions in *Eif2ak2*, *Mavs*, *Irf1*, *Irf3*, *Irf7*, *Irf9*, *Stat1* or the *Ifn-αβ* receptor (Fig. 2). These strains are viable and known to be hypersusceptible to RNA viruses, due to impaired innate immune responses. The luminescent reporter signal was slightly elevated during the early phase after infection with HCV-Cre in most EFT strains impaired in antiviral signalling as compared to EFT mice on a wild-type background (Fig. 2a–d). Between 20 and 40 days after infection there was a marked increase in the luciferase signal, particularly in IRF1 (eight-fold), IRF7 (16-fold) (Fig. 2c), IFN-αβR (20-fold) and STAT1 (40-fold) deficient mice (Fig. 2d) compared to non-transgenic littermate controls. The increased reporter signal, which presumably reflects a transient burst in viral replication and spread, eventually returned to background levels, possibly marking clearance of HCV-infected cells by the murine immune system. The elevated luminescent signal correlated with increases in serum HCV RNA levels at peak time points (Fig. 2e).

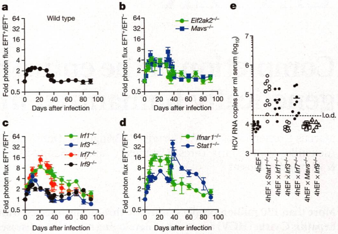

Figure 2 | Blunting of antiviral immune responses in mice expressing HCV entry factors augments HCV RNA replication. **a–d,** Bioluminescence kinetics of BiCre-Jc1-infected ROSA26-Fluc mice expressing all four HCV entry factors and entry-factor-negative controls with fully intact innate immune system (**a**) or impaired in EIF2AK2 or MAVS (**b**), IRF1, IRF3, IRF7 or IRF9 (**c**), and IFN-αβR or STAT1 (**d**). **e,** HCV RNA levels in the serum of 4hEF mice or 4hEF mice deficient in STAT1, IRF1, IRF3, IRF7, MAVS or IRF9 6 weeks after infection with BiCre-Jc1.

To validate that the elevated signal is indeed due to increased HCV RNA replication in mice with blunted antiviral immunity, we crossed cyclophilin A (CypA)-deficient mice (*Ppia*$^{-/-}$) to the EFT ROSA26-Fluc *Stat1*$^{-/-}$ background. CypA is a critical host factor for HCV RNA replication in human cells[15]. In *Ppia*$^{-/-}$ 4hEF ROSA26-Fluc *Stat1*$^{-/-}$ mice HCV RNA (Fig. 3c) and the luminescent reporter signal at peak times (day 31) was more than 60% lower than that of *Ppia*$^{+/+}$ and *Ppia*$^{+/-}$ mice (Fig. 3d), probably as a direct consequence of the murine PPIA deficiency. These data provide the first direct genetic evidence that CypA is a bona fide HCV replication factor *in vivo*. Furthermore, treatment of 4hEF *Stat1*$^{-/-}$ mice with an NS5A inhibitor for 3 weeks after infection suppressed HCV RNA loads to below the limit of detection (Fig. 3e), providing additional evidence that HCV RNA does indeed replicate in these mice.

Unambiguous detection of HCV antigens *in situ* is difficult. Therefore, we constructed a transgenic mouse line expressing a modified version of a previously described cell-based fluorescent reporter system[16] to visualize infection directly in the liver of infected mice (Supplementary Fig. 8). This highly sensitive reporter, the activity of which directly correlates with the level of HCV RNA replication, is based on cleavage of a blue fluorescent protein (TagBFP)–MAVS fusion protein by the HCV NS3-4A serine protease and translocation of the mitochondrially anchored fluorescent protein to the nucleus. The chimaeric TagBFP–nlsMAVS protein was widely expressed in the liver as assessed by histology and flow cytometry (Supplementary Fig. 8a, b). To quantify HCV protease-mediated nuclear re-localization accurately we subjected single hepatocyte suspensions to ImageStream X analysis. Adenovirus-mediated overexpression of NS3-4A activated the reporter in a majority of cells (Supplementary Fig. 9), demonstrating the functionality of the reporter *in vivo*. In EFT$^+$ but not EFT$^-$ (Fig. 3a) TagBFP–nlsMAVS transgenic mice HCV infection causes overlap of TagBFP fluorescence with the nuclear counterstain in approximately 0.2% of the cells (Fig. 3b). This low but HCV-specific reporter activation is probably due to the limited amounts of NS3-4A being translated from the incoming viral RNA, which cannot accumulate as a consequence of the abortive presumably single-cycle infection on a fully immunocompetent background. In contrast, crossing the EFT$^+$ TagBFP–nlsMAVS strain to a *Stat1*-null background increased the frequency of cells displaying HCV infection twofold over STAT1-positive controls (to 0.4%). Notably, whereas the reporter was activated only transiently

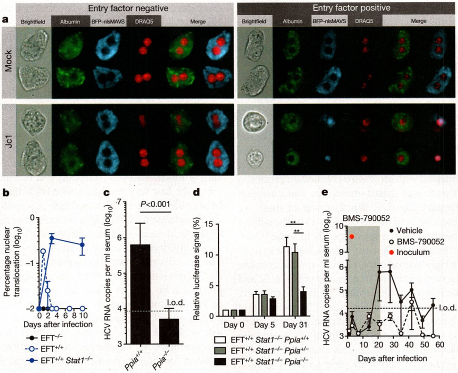

Figure 3 | Visualization and genetic and pharmacological interference with HCV infection. a, b, Quantification of murine hepatocytes actively replicating HCV in wild-type, 4hEF and 4hEF *Stat1*$^{-/-}$ mice as measured by the HCV NS3-4a-dependent cleavage of the TagBFP–nlsMAVS transgenic reporter construct by ImageStream X analysis. **c–e,** Longitudinal HCV RNA levels and luciferase signal in 4hEF *Stat1*$^{-/-}$ mice lacking PPIA (**c**, **d**), and longitudinal HCV RNA levels in 4hEF *Stat1*$^{-/-}$ mice treated with an HCV NS5A inhibitor (BMS-790052) for 20 days (**e**). Data shown are mean ± s.d. of $n = 10$–18 mice from two independent experiments. $^{**}P < 0.01$.

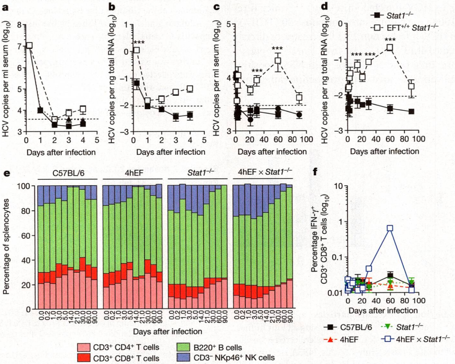

Figure 4 | HCV infection in 4hEF *Stat1*$^{-/-}$ mice leads to immune activation. a–d, HCV RNA copies in serum (**a**, **c**) and liver (**b**, **d**) of 4hEF *Stat1*$^{-/-}$ mice during early (**a**, **b**) or late (**c**, **d**) infection with Con1/Jc1. **e,** Relative frequencies of the indicated lymphocyte subsets in spleens of wild-type, 4hEF, *Stat1*$^{-/-}$ or *Stat1*$^{-/-}$ 4hEF mice isolated at the indicated time points after infection with Con1/Jc1. **f,** Analysis of liver-infiltrating IFN-γ-producing CD3^{+}CD8^{+} T cells of wild-type, 4hEF, *Stat1*$^{-/-}$ and 4hEF *Stat1*$^{-/-}$ mice after infection with Con1/Jc1. Data shown are mean ± s.d. of three independent experiments. $^{***}P < 0.001$.

in STAT1 sufficient mice, the signal persisted at least until day 10 in mice on a *Stat1*$^{-/-}$ background (Fig. 3b). Taken together, these results indicate that HCV can replicate in immunocompromised mice expressing human CD81 and OCLN and highlight the value of the model for studying HCV replication *in vivo*.

We further characterized viral kinetics in mice infected with a monocistronic cell-culture-derived HCV (HCVcc). HCV RNA rose approximately tenfold over the limit of quantification in serum (Fig. 4a, c) and liver (Fig. 4b, d) of EFT *Stat1*$^{-/-}$ mice as compared to non-transgenic *Stat1*$^{-/-}$ controls. Mice remained persistently infected for most of the observation period, with HCV RNA becoming nearly undetectable after 90 days (Fig. 4c, d). Sequence analysis of HCV RNA detected at late time points in mice infected with HCV-Cre revealed mutations in some viral genomes, but none was shared among the five mice that were analysed (Supplementary Fig. 12). Whether any of these are adaptive mutations that increase viral fitness *in vivo* will be the subject of future studies. HCV infection caused splenomegaly in some innate immune-deficient mice expressing HCV entry factors, but not in non-EFT mice (Supplementary Fig. 10a), indicative of HCV-specific immune activation. Indeed we observed increased frequencies of NK cells and subsequently B cells in the spleens of 4hEF *Stat1*$^{-/-}$ mice in the early and late phases of infection, respectively (Fig. 4e and Supplementary Fig. 10b). Consistent with results in humans and chimpanzees, primarily NK and IFN-γ-producing CD8^{+} T cells (Fig. 4f) infiltrated the livers of 4hEF *Stat1*$^{-/-}$ mice with a skewing towards an effector memory phenotype near the end of the time course (Supplementary Fig. 10c). These data indicate that HCV infection elicits cellular immune responses, albeit potentially confounded by the STAT1 deficiency, which may contribute to eventual viral clearance.

To determine whether primary hepatocytes in EFT mice on immunodeficient backgrounds were capable of producing infectious virions, sera collected at day 40 after infection were used to inoculate naive Huh-7.5 cells. Infectious virus was detected in sera of 4hEF mice deficient for STAT1, IRF1 and IRF7 (Fig. 5a), consistent with the increased levels of luminescent reporter activity (Fig. 2c, d) and HCV RNA load (Fig. 2e) in these strains. In HCV-infected 4hEF *Stat1*$^{-/-}$ mice titres reached approximately 100 tissue culture infectious dose 50 ($TCID_{50}$) per ml (Fig. 5b). Sera from HCV-infected non-EFT littermates, or directly acting antiviral (DAA)-treated 4hEF *Stat1*$^{-/-}$ or 4hEF *Stat1*$^{-/-}$ *Ppia*$^{-/-}$ mice collected at the same time points, did not yield NS5A-positive cells (data not shown), indicating *de novo* virus production rather than carry-over of the inoculum. To demonstrate that the lack of HCV NS5A-positive cells in the infectivity assay cannot simply be attributed to residual quantities of the DAA in circulation, we spiked the sera from the BMS-790052-treated animals with tissue-culture-produced BiCre-Jc1 virus (Fig. 5b) and found no inhibitory effect. Furthermore, in Huh-7.5 cells expressing an TagRFP–nlsMAVS reporter[16], infection with sera from infected mice resulted in re-localization of the reporter signal whereas inoculation with sera from non-infected, DAA-treated or *Ppia*$^{-/-}$ mice did not (Supplementary Fig. 11). These data are in accordance with previous *in vitro* observations demonstrating that mouse hepatoma cells support late stages of the HCV life cycle[17].

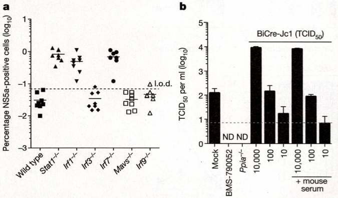

Figure 5 | Evidence for production of infectious particles. a, *Stat1*$^{-/-}$, *Irf1*$^{-/-}$, *Irf3*$^{-/-}$, *Irf7*$^{-/-}$, *Irf9*$^{-/-}$ and *Mavs*$^{-/-}$ mice expressing all four human HCV entry factors were infected with BiCre-Jc1. Sera were collected 6 weeks after infection and were used to infect naive Huh-7.5 cells. NS5A staining was performed 72 h after infection and the frequency of HCV antigen-bearing cells quantified by flow cytometry. **b**, HCV infectious particles released into the serum of 4hEF *Stat1*$^{-/-}$ mice, 4hEF *Stat1*$^{-/-}$ mice lacking PPIA or 4hEF *Stat1*$^{-/-}$ mice treated with BMS-790052 for 20 days as determined by limiting dilution assay. Data shown are mean ± s.d. of four independent experiments. ND, not detectable.

This study represents an important step forward in developing an animal model for HCV infection and immunity. To our knowledge this is the first time that the entire HCV life cycle has been recapitulated in inbred mice with inheritable susceptibility to HCV. Previously developed xenotransplant mouse models can also be infected with HCV. However, these models are hampered by intra- and inter-experimental variability, donor-to-donor variability, low throughput, and high costs. The inbred mouse model presented here can overcome many of these challenges, is amendable to genetic manipulations and can be used for preclinical assessment of the efficacy of antiviral drug candidates and entry inhibitors. To study unperturbed HCV-specific immune responses and HCV-associated pathogenesis it will be necessary to establish persistent HCV infection in fully immunocompetent mice. By harnessing the remarkable genetic plasticity of HCV it may be possible to select for viral variants that replicate more robustly in sufficiently immunocompromised rodent strains. High titre sera could subsequently be passaged through progressively more immunocompetent hosts to produce this outcome. Future studies will address the capacity of different, genetically diverse HCV genotypes to establish chronicity in genetically humanized mice.

METHODS SUMMARY

Mice. Construction of all mice expressing HCV entry factors under the control of a liver-specific albumin promoter or CAGGS-TagBFP–nlsMAVS and the source of all other mouse mutant strains is described in the Methods. Mice were bred and maintained at the Comparative Bioscience Center of The Rockefeller University according to guidelines established by the Institutional Animal Committee.

Hepatitis C virus. Plasmids encoding chimaeric HCV genomes, including Jc1, Con1-Jc1 and bicistronic HCV genomes expressing Cre, were linearized with XbaI and *in vitro* transcribed. RNA was electroporated into Huh-7.5 cells and infectious virus was collected from supernatants 48–72 h after transfection[18].

RT–PCR quantification of HCV RNA. Total RNA was isolated from mouse brain, liver and sera using the RNAeasy kit (Qiagen). HCV genome copy number was quantified by one-step RT–PCR.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions M.D. and J.A.H. planned and performed experiments and contributed to writing the manuscript. B.M.D., R.N.L., W.C.B., T.F., A.V. and M.T.C. performed the experimental work; T.K., T.S., S.A. and M.L. provided reagents. C.M.R. provided laboratory infrastructure, space, reagents, advice and edited the manuscript. A.P. planned and performed experiments and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.P. (aploss@princeton.edu) or C.M.R. (ricec@rockefeller.edu).

LETTER

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Pathogen blocks host death receptor signalling by arginine GlcNAcylation of death domains

Shan Li[1,2]*, Li Zhang[2,3]*, Qing Yao[1,2], Lin Li[2], Na Dong[2], Jie Rong[4], Wenqing Gao[2], Xiaojun Ding[2], Liming Sun[2], Xing Chen[4], She Chen[2] & Feng Shao[2]

The tumour necrosis factor (TNF) family is crucial for immune homeostasis, cell death and inflammation. These cytokines are recognized by members of the TNF receptor (TNFR) family of death receptors, including TNFR1 and TNFR2, and FAS and TNF-related apoptosis-inducing ligand (TRAIL) receptors[1]. Death receptor signalling requires death-domain-mediated homotypic/heterotypic interactions between the receptor and its downstream adaptors, including TNFR1-associated death domain protein (TRADD) and FAS-associated death domain protein (FADD)[2]. Here we discover that death domains in several proteins, including TRADD, FADD, RIPK1 and TNFR1, were directly inactivated by NleB, an enteropathogenic *Escherichia coli* (EPEC) type III secretion system effector known to inhibit host nuclear factor-κB (NF-κB) signalling[3,4]. NleB contained an unprecedented *N*-acetylglucosamine (GlcNAc) transferase activity that specifically modified a conserved arginine in these death domains (Arg 235 in the TRADD death domain). NleB GlcNAcylation (the addition of GlcNAc onto a protein side chain) of death domains blocked homotypic/heterotypic death domain interactions and assembly of the oligomeric TNFR1 complex, thereby disrupting TNF signalling in EPEC-infected cells, including NF-κB signalling, apoptosis and necroptosis. Type-III-delivered NleB also blocked FAS ligand and TRAIL-induced cell death by preventing formation of a FADD-mediated death-inducing signalling complex (DISC). The arginine GlcNAc transferase activity of NleB was required for bacterial colonization in the mouse model of EPEC infection. The mechanism of action of NleB represents a new model by which bacteria counteract host defences, and also a previously unappreciated post-translational modification.

EPEC contains several type-III-secreted effectors, including NleC/D[5–8], Tir[9,10], NleE and NleB[3,4,11,12], all of which can inhibit host NF-κB signalling and pro-inflammatory cytokine production. Among these effectors, NleB is required for virulence *in vivo*[13–15]. NleB homologues are present in *Salmonella* and pathogenic *E. coli* strains[16]. Consistent with previous studies[4,9], the expression of NleB (E2348C_3231) in HeLa cells selectively blocked TNF-α but not interleukin (IL)-1β activation of NF-κB signalling (Supplementary Fig. 1a, b). TNF-α-induced IκB-α phosphorylation and degradation were both severely inhibited (Supplementary Fig. 1c). NleB blocked TRAF2- but not TRAF6-induced NF-κB activation (Supplementary Fig. 2a). TNF-α- but not IL-1β-stimulated TAK1 phosphorylation, downstream of the receptor and TRAF complex, was diminished by NleB (Supplementary Fig. 2b). TNFR can also induce apoptosis or necroptosis (in cells deficient in caspase activity), playing an important role in microbial infection and inflammation[17]. NleB efficiently blocked TNF-α plus cycloheximide-induced 293T cell apoptosis (Fig. 1a), and also blocked necroptosis of HeLa cells stably expressing the RIP3 protein kinase (HeLa-RIP3 cells) (Fig. 1b). This agrees with NleB targeting upstream of TAK1, as TAK1 is not required for TNF-α-induced cell death[18]. Thus, NleB differs from other NF-κB-targeting effectors and can block both NF-κB signalling and TNF-α-induced cell death.

A yeast two-hybrid screen of a HeLa complementary DNA library identified a cDNA clone encoding the death domain of TRADD, a universal component of the TNFR1 but not IL-1R complex[19–21]. NleB also interacted with full-length TRADD, but not with components of the TAK1 and IκB kinase (IKK) complexes; no interaction occurred between NleE and TRADD (Fig. 1c). Endogenous TRADD was readily precipitated by NleB (Supplementary Fig. 3a). The TRADD death domain (residues 195–312) was required and sufficient for precipitation by enhanced green fluorescent protein (eGFP)–NleB (Fig. 1d and Supplementary Fig. 3b). Similar to that observed with TNF-α stimulation, NleB completely abolished TRADD overexpression-induced NF-κB activation (Fig. 1e) and apoptosis in 293T cells (Fig. 1f and Supplementary Fig. 4a). Thus, NleB can target TRADD and disrupt multiple signalling pathways downstream of TNFR1.

NleB did not affect TRADD stability or turnover (Supplementary Fig. 5a). TRADD contains an amino-terminal TRAF2-binding domain and a carboxy-terminal death domain. The TRADD death domain oligomerizes with itself and also with death domains of TNFR1 and FADD[22]. Death-domain-mediated TRADD recruitment to TNFR1 initiates TNF-α signalling, and TRADD–FADD complex formation triggers apoptosis[23]. TRADD readily precipitated TNFR1c (the intracellular death-domain-containing region) from 293T cells, and this was abolished by NleB (Supplementary Fig. 5b). A similar blocking effect was observed in TRADD–FADD but not in TRADD–TRAF2 co-immunoprecipitation (Supplementary Fig. 5c, 6). TRADD oligomerization is crucial for TNFR1 complex assembly[22]. TRADD or TRADD death domain expressed in 293T cells appeared as a large oligomer on a blue native polyacrylamide gel electrophoresis (PAGE) gel, and NleB co-expression shifted TRADD and also the TRADD death domain to a lower molecular mass position that roughly corresponded to the monomer form (Fig. 2a). Consistently, NleB inhibited the recruitment of TRADD, TRAF2, HOIP, SHAPPIN, HOIL-1L and ubiquitinated RIPK1 to TNFR1 (Fig. 2b). Thus, NleB inactivates the TRADD death domain and disrupts homotypic/heterotypic death domain interactions and the TNFR1 complex assembly.

In these TRADD oligomerization and binding assays, the amount of NleB was considerably lower than those of TRADD, indicating that NleB may act in a catalytic manner on the TRADD death domain. Mass spectrometry analysis identified a 203-dalton (Da) mass increase on TRADD death domain co-expressed with NleB in either *E. coli* (Supplementary Fig. 7) or 293T cells (Fig. 2c), whereas control TRADD death domain showed the theoretic molecular mass. The 203-Da mass increase indicated a GlcNAc modification. Consistently, recombinant NleB efficiently transferred ^{3}H-GlcNAc, but not ^{3}H-glucose, from radiolabelled sugar donors onto purified TRADD (Fig. 2d). Cold UDP-GlcNAc, but not UDP-GalNAc, UDP-glucose, UDP-galactose and UDP-GlcA,

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[1]College of Biological Sciences, China Agricultural University, Beijing 100094, China. [2]National Institute of Biological Sciences, Beijing 102206, China. [3]Graduate School of Chinese Academy of Medical Sciences and Beijing Union Medical College, Beijing 100730, China. [4]Beijing National Laboratory for Molecular Sciences, Department of Chemical Biology, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China.

*These authors contributed equally to this work.

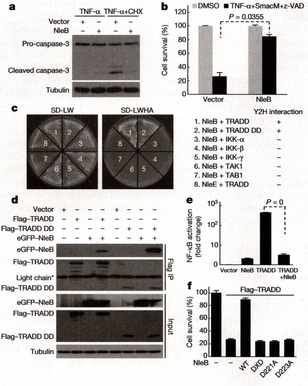

Figure 1 | NleB blocks TNF-α signalling by directly targeting the TRADD death domain. **a**, Effects of NleB transfection on TNF-α-induced caspase-3 activation in 293T cells. CHX, cycloheximide. **b**, Vector- or NleB-transfected HeLa-RIP3 cells were stimulated with TNF-α plus Smac mimetic (SmacM) and z-VAD, and cell viability was determined by measuring ATP levels. DMSO, dimethylsulphoxide. **c**, Yeast strain was transformed with indicated plasmid combinations (bait plus prey) to assay the interaction of NleB with TRADD or other components downstream of TNFR. Yeast strains were grown on SD-LW (non-selective) and SD-LWHA (selective) media. DD, death domain; Y2H, yeast two-hybrid. **d**, Co-immunoprecipitation of NleB with TRADD and TRADD death domain in 293T cells. **e**, NF-κB luciferase activity of NleB- and/or TRADD-transfected 293T cells is shown as fold change normalized to that in vector-transfected cells. **f**, Viability of NleB- and/or TRADD-transfected 293T cells was determined by measuring ATP levels. $P < 0.0001$ for all (TRADD plus NleB versus TRADD plus DXD, versus TRADD plus Asp221Ala, and versus TRADD plus Asp223Ala). For **b**, **e** and **f**, $n = 3$; data are mean ± s.d.; P value determined by Student's t-test. Data in **a**, **c** and **d** are representative from at least three repetitions.

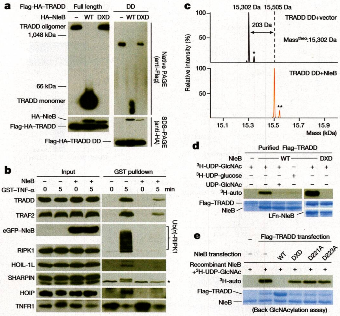

Figure 2 | NleB GlcNAcylates the TRADD death domain and disrupts its oligomerization. **a**, Effects of NleB transfection on TRADD oligomerization in 293T cells. Shown are immunoblots of cell lysates loaded onto native (top) and SDS–PAGE (bottom) gels. HA, haemagglutinin; WT, wild type. **b**, Effects of NleB transfection on TNFR1 complex formation in HeLa cells. Ub(n)-RIPK1 denotes polyubiquitinated RIPK1; asterisk marks a nonspecific band. GST, glutathione S-transferase. **c**, Electrospray ionization (ESI)-mass spectrometry determination of the total mass of TRADD death domain immunopurified from NleB- or vector-transfected 293T cells. Single and double asterisks denote the acetylated TRADD death domain. **d**, *In vitro* ^{3}H-UDP-GlcNAc labelling of TRADD by recombinant NleB. LFn-NleB refers to NleB protein fused C-terminally to the N-terminal domain of anthrax lethal factor. **e**, TRADD immunopurified from NleB-transfected 293T cells was subjected to further *in vitro* ^{3}H-UDP-GlcNAc labelling by recombinant NleB. Data in **a**, **b**, **d** and **e** are representative from at least three repetitions.

abolished ^{3}H-GlcNAc labelling of TRADD by NleB (Fig. 2d and Supplementary Fig. 8). TRADD purified from NleB-expressing cells was resistant to further *in vitro* ^{3}H-GlcNAc labelling by recombinant NleB in the 'back glycosylation' assay[24] (Fig. 2e), confirming the complete modification of TRADD in NleB-expressing 293T cells. Thus, NleB is a glycosyltransferase that GlcNAcylates the TRADD death domain.

Extensive mutagenesis of potentially catalytic residues identified NleB Asp221Ala and Asp223Ala mutants that lost the activity of inhibiting TNF-α–NF-κB signalling (Supplementary Fig. 9). Interestingly, the GT-A family of glycosyltransferases[25], including the large clostridial toxins that modify Rho/Ras small GTPases[26], feature a catalytic Asp-X-Asp (DXD) motif that coordinates manganese (Mn^{2+}) and/or the sugar donor. The NleB(Asp221Ala/Asp223Ala) (DXD) mutant could not block the death-domain-mediated TRADD interaction with TNFR1c and FADD (Supplementary Fig. 5b, c) or TRADD self-oligomerization (Fig. 2a). Recombinant NleB DXD mutant exhibited no catalytic activity of GlcNAcylating TRADD (Fig. 2d); these two aspartate residues were required for NleB interaction (Supplementary Fig. 3a) and modification of TRADD in transfected 293T cells (Fig. 2e), as well as for inhibiting TRADD-induced apoptosis (Fig. 1f and Supplementary Fig. 4a). Concurrent to our analysis, another study[27] also proposes that NleB is a GT-A-type glycosyltransferase according to fold recognition prediction. This study identifies glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a host binding partner of NleB. In *O*-GlcNAc antibody blotting, ^{3}H-GlcNAc labelling and mass spectrometric assays, GAPDH was not glycosylated by NleB *in vitro* and *in vivo* (Supplementary Fig. 10). GAPDH binding may represent a regulatory mechanism of NleB function, or be a target of NleB homologue. EPEC contains an NleB paralogue known as NleB2 (E2348C_1041) and an NleB homologue, SseK1, is present in *Salmonella enterica* serovar Typhimurium. Although SseK1 functioned comparably to NleB in inhibiting TNF-α–NF-κB signalling and GlcNAcylating the TRADD death domain, NleB2 showed a lower enzymatic activity and only a fraction of TRADD was modified by NleB2 in 293T cells (Supplementary Fig. 11).

Protein *O*-GlcNAcylation is an *O*-linked GlcNAc modification generally occurring on serine/threonine residues of intracellular proteins. *O*-GlcNAcylation is abundant from bacteria to mammals, and has been proposed to be a phosphorylation-like modification in regulating transcription or signalling in response to nutrients and cellular stresses[28,29]. The 203-Da mass increase indicates a single GlcNAc modification to the TRADD death domain by NleB (Fig. 2c and Supplementary Fig. 7a). To pinpoint the exact modification site, mutagenesis analyses were first used given that mapping the *O*-GlcNAcylation site by the conventional collision-assisted dissociation (CAD) mass spectrometry is technically difficult owing to the labile nature of GlcNAc modification[29]. Surprisingly, no single mutation in all the ten serine/threonine residues in the TRADD death domain disrupted its modification by NleB (Supplementary Fig. 12). The ST10A mutant (all ten serine/threonine residues

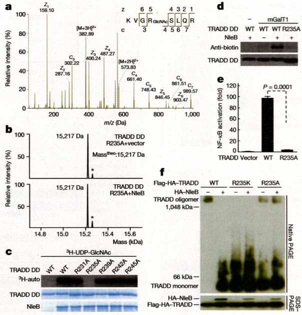

Figure 3 | NleB GlcNAcylates Arg 235 that is required for TRADD function. **a**, ETD-tandem mass spectrum of a triply charged Arg-235-containing tryptic peptide from NleB-modified TRADD death domain in bacteria. The fragmentation patterns that generate the observed *c* and *z* ions are illustrated along the peptide sequence shown on top of the spectrum. **b**, ESI-mass spectrometry determination of the total mass of TRADD arginine mutants immunopurified from NleB- or vector-transfected 293T cells. All arginine residues in the TRADD death domain were individually mutated into alanine (also see Supplementary Fig. 17). Asterisk denotes the acetylated TRADD death domain Arg235Ala mutant. **c**, *In vitro* ^{3}H-UDP-GlcNAc labelling of TRADD death domain arginine mutants by recombinant NleB. **d**, TRADD death domain or its Arg235Ala mutant immunopurified from NleB-transfected 293T cells was subjected to chemoenzymatic UDP-GalNAz labelling followed by reaction with an alkyne-biotin derivative. **e**, **f**, TRADD or its Arg 235 mutants were expressed in TRADD-knockdown 293T cells. **e**, NF-κB luciferase activity (fold change) is normalized to that in vector-transfected cells ($n = 3$; mean ± s.d.; Student's *t*-test). Data in **c**, **d** and **f** are representative from at least three repetitions.

were mutated simultaneously) was still GlcNAcylated to a considerable extent (Supplementary Fig. 12). Thus, NleB-induced modification may not be a canonical *O*-GlcNAcylation. The NleB-modified TRADD death domain (Supplementary Fig. 13) was then subjected to in-depth mass spectrometry analyses. Among the 13 tryptic peptides that cover the large majority of the TRADD death domain sequence, two overlapping ones (232-KVGRSLQR-239 and 233-VGRSLQR-239) showed a 203-Da mass increase (Supplementary Fig. 14). Electron-transfer dissociation (ETD) tandem mass spectrometry, which works better in preventing loss of the labile GlcNAc during peptide fragmentation[29], revealed that the 203-Da mass increase occurred on Arg 235 (Fig. 3a). When the Arg-235-containing tryptic peptide was purified and sequentially digested by proteinase K and carboxypeptidase, the GlcNAc-modified dipeptide (234-Gly-Arg-235) and even Arg 235 alone were identified by higher-energy collision dissociation tandem mass spectrometry (Supplementary Fig. 15). When the modification was performed in cells metabolically labelled with 2-(acetyl-d_3-amino)-2-deoxy-1,3,4,6-tetra-*O*-acetyl-D-glucopyranose (Ac$_4$GlcNAc-d_3), a mass increase of 206 Da, corresponding to modification by the deuterium-labelled GlcNAc, occurred on Arg 235 in the TRADD death domain (Supplementary Fig. 16).

NleB GlcNAcylation of TRADD Arg 235 was supported by four additional evidences. Arg235Ala and Arg235Lys mutations in the TRADD death domain abolished the GlcNAc modification by NleB in 293T cells, whereas mutation in any of the other ten arginine residues had no such effect (Fig. 3b and Supplementary Fig. 17). The purified TRADD(Arg235Ala) mutant resisted NleB-catalysed *in vitro* GlcNAcylation (Fig. 3c). When chemoenzymatic labelling of the terminal GlcNAc was used to detect GlcNAcylation, the wild-type TRADD

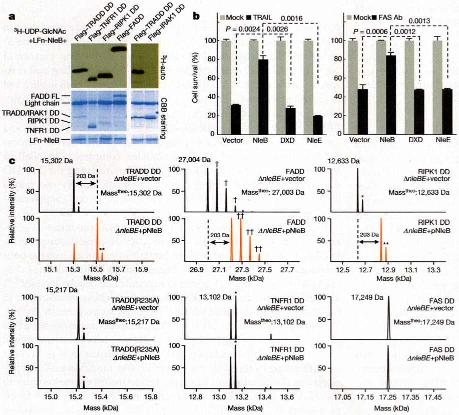

Figure 4 | NleB GlcNAcylates FADD and other death domain proteins, and blocks FAS- and TRAIL-induced apoptosis. **a**, *In vitro* ^{3}H-UDP-GlcNAc labelling of indicated death domains or death-domain-containing proteins by recombinant NleB. Data represent at least three repetitions. **b**, NleB-, NleE- or vector-transfected HeLa cells were stimulated with FAS antibody (Ab) or TRAIL, and cell viability was determined by measuring ATP levels ($n = 3$; mean ± s.d.; Student's *t*-test). **c**, ESI-mass spectrometry determination of the total mass of indicated death domain proteins purified from EPEC-infected 293T cells. Δ*nleBE*, NleB and NleE double-deletion EPEC E2348/69 strain; pNleB, NleB rescue plasmid. Dagger symbols denote the phosphorylated FADD; asterisks mark the corresponding acetylated proteins.

death domain from NleB-expressing 293T cells was readily labelled with *N*-azidoacetylgalactosamine (GalNAz) by an engineered galactosyltransferase (Tyr289Leu mutant of GalT1), whereas no evident GalNAz labelling occurred on TRADD(Arg235Ala) (Fig. 3d). Lastly, NleB-catalysed GlcNAc modification was not reversed by *O*-GlcNAcase, β-*N*-acetylglucosaminidase and β-*N*-acetylhexosaminidase$_f$ (a recombinant protein fusion of β-*N*-acetylhexosaminidase and maltose binding protein) (Supplementary Fig. 18), enzymes that are capable of removing *O*-linked or terminal GlcNAc.

TRADD-knockdown 293T cells were generated (Supplementary Fig. 19a). Knockdown of endogenous TRADD minimized its hetero-oligomerization and interference with transfected TRADD mutants. In contrast to wild-type TRADD, expression of TRADD(Arg235Ala) in TRADD-knockdown cells was largely deficient in activating the NF-κB signalling (Fig. 3e and Supplementary Fig. 19b). Consistently, lysine or alanine substitution of Arg 235 in TRADD completely or nearly completely disrupted TRADD self-oligomerization, and the residual Arg235Ala oligomer was insensitive to further NleB expression (Fig. 3f). Thus, Arg 235 is important for TRADD function and activity.

Arg 235 is conserved in one-third of the total of more than 30 death-domain-containing proteins in humans[22] (Supplementary Fig. 20), including FADD, TNFR1, RIPK1, FAS and death receptor-3/4/5 (DR3/4/5) that function in death receptor signalling. NleB completely modified the death domains of TNFR1 and RIPK1 as well as full-length FADD in bacteria (Supplementary Fig. 21) or 293T cells (Supplementary Fig. 22); a considerable portion of FAS death domain was also GlcNAc-modified. Death domains of MYD88 and IRAK1 devoid of the conserved arginine were not modified by NleB (Supplementary Fig. 22). In the ^{3}H-GlcNAc labelling assay, FADD and death domains of TNFR1 and RIPK1 were GlcNAcylated by recombinant NleB, with efficiency comparable to that of the TRADD death domain (Fig. 4a). Induction of apoptosis by FAS ligand (FasL) and TRAIL require death-domain-mediated FADD interaction with the receptors and formation of a caspase-8-containing death-inducing signalling complex (DISC). Consistently, anti-FAS antibody and TRAIL-stimulated HeLa cell apoptosis was inhibited by NleB, but not by the GlcNAc-transferase-deficient DXD mutant or the NleE effector (Fig. 4b).

293T cells expressing various death domains or death domain proteins were infected with an NleB-proficient or -deficient EPEC strain. FADD, RIPK1 death domain and a portion of TRADD death domain from NleB-positive infection showed a 203-Da mass increase (Fig. 4c). Modification of these proteins was not observed with the NleB-deficient strain. Importantly, no GlcNAc modification occurred on TRADD(Arg235Ala) (Fig. 4c). Consistent with these observations, expression of NleB, but not the DXD mutant, in a NleB/E double-deletion EPEC strain blocked p65 nuclear translocation in infected mouse embryonic fibroblast (MEF) cells (Fig. 5a and Supplementary Fig. 23a). GlcNAc-transferase-active NleB inhibited apoptosis in EPEC-infected HeLa cells after stimulation by TNF-α, FasL or TRAIL (Fig. 5b and Supplementary Fig. 4b). Type-III-delivered NleB, but not the DXD mutant, also effectively blocked TNF-α-induced necroptosis in HeLa-RIP3 or HT-29 cells (Supplementary Fig. 24). Furthermore, TNF-α-induced recruitment of TRADD, TRAF2, SHARPIN and ubiquitinated RIPK1 to TNFR1 as well as TRAIL-induced DISC formation were both disrupted by EPEC expressing the catalytically active NleB, and disruption of DISC formation was more severe (Fig. 5c and Supplementary Fig. 23b). When expressed in the type III secretion-deficient Δ*escN* strain, NleB did not affect cell death and death receptor complex assembly (Fig. 5a–c and Supplementary Figs 23b and 24).

Deletion of *nleB* from *Citrobacter rodentium* results in severely reduced bacterial colonization in infected mice[13]. *C. rodentium* NleB (NleBc) inhibited TNF-α signalling to an extent comparable to that of EPEC NleB (Supplementary Fig. 25a); NleBc also GlcNAcylated several death domain proteins, including FADD, TNFR1 and RIPK1, despite a slightly different substrate preference (Supplementary Fig. 25b). In *C.-rodentium*-inoculated C57BL/6 mice, the Δ*nleB* mutant showed a significantly reduced colonization compared to the wild-type strain,

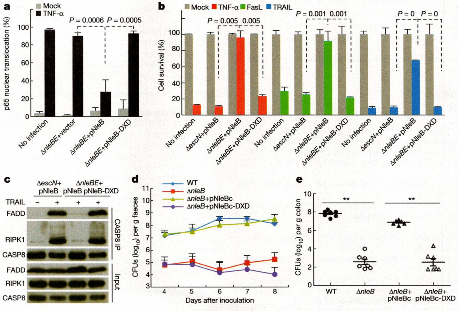

Figure 5 | Disrupting several death receptor pathways by NleB GlcNAc transferase activity that is required for bacterial colonization *in vivo*. **a**, MEF cells infected with indicated EPEC strains were stimulated with TNF-α, and statistics of cells with nuclear-localized p65 are shown. Representative fluorescence images are in Supplementary Fig. 23a. **b**, HeLa cells infected with indicated EPEC strains were stimulated with TNF-α, FasL or TRAIL, and cell viability was determined by measuring ATP levels. For **a** and **b**, $n = 3$; mean ± s.d. **c**, Lysates of EPEC-infected HeLa cells stimulated with TRAIL were subjected to anti-caspase-8 (CASP8) immunoprecipitation. Data represent at least three repetitions. **d**, **e**, Five–six-week-old C57BL/6 mice were oral gavaged with indicated *C. rodentium* DBS100 strains. Viable stool bacterial counts (**d**), measured at indicated points after inoculation, are shown as mean ± s.e.m. of log$_{10}$ colony-forming units (CFU) per gram faeces, and bacterial colonization of the intestine 8 days after infection (**e**) is shown as the mean ± s.e.m. of log$_{10}$ CFU per gram colon ($n > 6$). $^{**}P < 0.01$ (Student's *t*-test). pNleB(c) and pNleB(c)-DXD are rescue plasmids expressing wild-type and the DXD mutant of NleB(c), respectively.

demonstrated by colony-forming units of bacteria recovered from stool samples and colons from infected mice (Fig. 5d, e). Complementation of Δ*nleB* with NleBc or EPEC NleB, but not the DXD mutant, recovered stool counts to the level of the wild-type strain and restored bacterial colonization in the intestinal tract of mice (Fig. 5d, e and Supplementary Fig. 26). Thus, the arginine GlcNAc transferase activity of NleB is crucial for bacterial colonization and virulence in mice.

We discover that the EPEC type III effector NleB targets several death-domain-containing proteins in TNFR, FAS and TRAIL death receptor complexes to block host cell death. NleB probably functions as part of a network of effectors during EPEC interaction with the host. NleB is the first bacterial virulence factor hijacking the death receptor complex. Such distinguished feature may account for the unique virulence activity of NleB[13–15]. TRADD and FADD are central adaptors in death receptor signalling. TRADD also mediates Toll-like receptor signalling in macrophages[19–21], suggesting an even broader function of NleB in EPEC modulation of host defences. NleB modifies its death domain targets by GlcNAcylation on a conserved arginine. Discovering arginine GlcNAcylation is conceptually unexpected despite one early preliminary study proposing such modification on a corn protein[30]. The labile GlcNAc modification is refractory to conventional mass-spectrometry-based proteomic identifications that are performed in the absence of an arginine GlcNAcylation hypothesis. Thus, arginine GlcNAcylation might be widely used and represent a previously unappreciated mechanism in signalling.

METHODS SUMMARY

The EPEC E2348/69 Δ*nleB/E* SC3909 strain (ΔIE2::*kan* and *nleBE IE6::tet*)[3] was used for cell culture infection. A single bacterial colony was inoculated into 0.5 ml of LB medium and statically cultured overnight at 37 °C. Bacterial cultures were then diluted by 1:40 in DMEM supplemented with 1 mM isopropyl-β-D-thiogalactoside (IPTG) and cultured for an additional 4 h at 37 °C in the presence of 5% CO_2. Infection was performed at a multiplicity of infection of 200:1 in the presence of 1 mM IPTG for 2 h. Cells were washed four times with PBS and bacteria were killed by 200 $\mu g\ ml^{-1}$ gentamicin. To assay NleB-induced modification, 293T cells were transfected with pCS2-Flag-TRADD death domain plasmids 24 h before infection. z-VAD (Sigma) (20 μM) was added to inhibit apoptosis. Lysates of infected cells were subjected to anti-Flag immunoprecipitation to purify the TRADD death domain for mass spectrometry analysis. All independent experiments carried out in this study and indicated in the figure legends were biological replicates.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions S.L. and L.Z. performed most experiments, assisted by Q.Y., N.D. and W.G. L.L., X.D. and S.C. performed mass spectrometry analyses and S.C. analysed the data. L.S., J.R. and X.C. contributed reagents and analytic tools. S.L., L.Z. and F.S. analysed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.C. (chenshe@nibs.ac.cn) or F.S. (shaofeng@nibs.ac.cn).

LETTER

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A type III effector antagonizes death receptor signalling during bacterial gut infection

Jaclyn S. Pearson[1], Cristina Giogha[1], Sze Ying Ong[1], Catherine L. Kennedy[1], Michelle Kelly[1], Keith S. Robinson[2], Tania Wong Fok Lung[1], Ashley Mansell[3], Patrice Riedmaier[1], Clare V. L. Oates[1], Ali Zaid[1], Sabrina Mühlen[1], Valerie F. Crepin[2], Olivier Marches[4], Ching-Seng Ang[5], Nicholas A. Williamson[5], Lorraine A. O'Reilly[6,7], Aleksandra Bankovacki[6], Ueli Nachbur[6], Giuseppe Infusini[6], Andrew I. Webb[6,7], John Silke[6,7], Andreas Strasser[6,7], Gad Frankel[2] & Elizabeth L. Hartland[1,8]

Successful infection by enteric bacterial pathogens depends on the ability of the bacteria to colonize the gut, replicate in host tissues and disseminate to other hosts. Pathogens such as *Salmonella*, *Shigella* and enteropathogenic and enterohaemorrhagic (EPEC and EHEC, respectively) *Escherichia coli* use a type III secretion system (T3SS) to deliver virulence effector proteins into host cells during infection that promote colonization and interfere with antimicrobial host responses[1–3]. Here we report that the T3SS effector NleB1 from EPEC binds to host cell death-domain-containing proteins and thereby inhibits death receptor signalling. Protein interaction studies identified FADD, TRADD and RIPK1 as binding partners of NleB1. NleB1 expressed ectopically or injected by the bacterial T3SS prevented Fas ligand or TNF-induced formation of the canonical death-inducing signalling complex (DISC) and proteolytic activation of caspase-8, an essential step in death-receptor-induced apoptosis. This inhibition depended on the *N*-acetylglucosamine transferase activity of NleB1, which specifically modified Arg 117 in the death domain of FADD. The importance of the death receptor apoptotic pathway to host defence was demonstrated using mice deficient in the FAS signalling pathway, which showed delayed clearance of the EPEC-like mouse pathogen *Citrobacter rodentium* and reversion to virulence of an *nleB* mutant. The activity of NleB suggests that EPEC and other attaching and effacing pathogens antagonize death-receptor-induced apoptosis of infected cells, thereby blocking a major antimicrobial host response.

Members of the death receptor family such as FAS and TNFR1 contain an intracellular death domain and require caspase-8 for the activation of effector caspases and consequent cell death by the extrinsic apoptotic pathway[4,5]. Within seconds of engagement by Fas ligand (FasL), pre-associated FAS trimers undergo multimerization and recruit the death-domain-containing adaptor protein FADD through heterotypic death domain interactions[5]. FADD then recruits pro-caspase-8 forming the death-inducing signalling complex (DISC), whereupon caspase-8 undergoes conformational change, auto-proteolytic processing and release from the DISC to allow cleavage of cytosolic substrates, including pro-caspase-3 and Bid[5]. Similarly, upon stimulation by TNF, TNFR1 recruits the adaptor protein TRADD and the receptor kinase, RIPK1, leading to NF-κB activation. Alternatively, cell death may be induced through TNFR1 internalisation and formation of a secondary complex that contains TRADD/RIPK1/FADD and caspase-8 (refs 6, 7).

Previously, we reported that the EPEC T3SS effector NleB1 inhibited IκB degradation in cells upon stimulation with TNF but not IL-1β, suggesting that NleB1 interfered with death receptor signalling[8]. To identify host cell binding partners of NleB1, we screened a yeast two-hybrid complementary DNA (cDNA) library derived from HeLa cells using NleB1 as bait. FADD and RIPK1, were recovered several times.

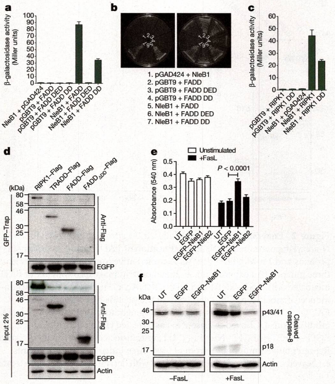

Figure 1 | NleB1 binds death domain proteins and inhibits caspase-8 activation. **a**, Yeast two-hybrid analysis of protein–protein interactions in *S. cerevisiae* PJ69-4A. Results are mean ± s.e.m. β-galactosidase activity from at least three independent experiments performed in triplicate. DD, death domain; DED, death effector domain. **b**, Growth of *S. cerevisiae* PJ69-4A on medium to select for protein–protein interactions (left panel) or plasmid maintenance (right panel). Representative images from at least three independent experiments. **c**, Yeast two-hybrid analysis of protein interactions in *S. cerevisiae* PJ69-4A. Results are mean ± s.e.m. β-galactosidase activity from at least three independent experiments performed in triplicate. **d**, GFP-Trap of EGFP–NleB1 and detection of FADD–Flag, TRADD–Flag and RIPK1–Flag in HEK293T cells. Actin, loading control. Representative immunoblot from at least three independent experiments. **e**, MTT reduction in HeLa cells expressing EGFP, EGFP–NleB1 or EGFP–NleB2. UT, untransfected. Results are the mean ± s.e.m. of absorbance at 540 nm from three independent experiments performed in triplicate. *$P < 0.0001$, unpaired, two-tailed *t*-test. **f**, Cleaved caspase-8 in HeLa cells expressing EGFP or EGFP–NleB1. p43/41 and p18 are products of processed caspase-8. UT, untransfected. Representative immunoblot from at least three independent experiments.

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[1] Department of Microbiology and Immunology, University of Melbourne, Victoria 3010, Australia. [2] MRC Centre for Molecular Bacteriology and Infection, Department of Life Sciences, Imperial College, London SW7 2AZ, UK. [3] Centre for Innate Immunity and Infectious Diseases, Monash Institute of Medical Research, Victoria 3010, Australia. [4] Centre for Immunology and Infectious Disease, Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London E1 2AT, UK. [5] Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Victoria 3010, Australia. [6] The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia. [7] Department of Medical Biology, University of Melbourne, Victoria 3010, Australia. [8] Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia.

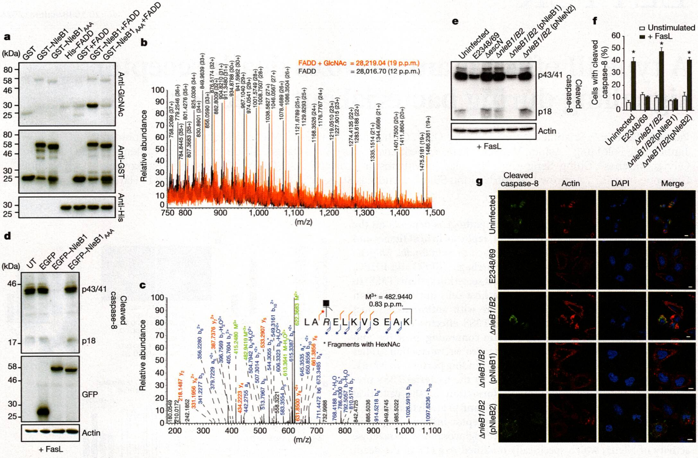

Figure 2 | Enzymatic activity of NleB1. a, *In vitro* assay for NleB1 *N*-acetylglucosamine (GlcNAc) modification of FADD using recombinant proteins and 1 mM UDP-GlcNAc. Representative immunoblot from at least three independent experiments. **b,** Intact protein mass spectrometry of FADD incubated with GST–NleB1 and UDP-GlcNAc. **c,** High resolution collision-induced dissociation (CID) spectrum of the peptide corresponding to FADD$^{115-125}$. The * denotes diagnostic fragment ions that carry the GlcNAc modification. **d,** Cleaved caspase-8 in FasL-treated HeLa cells expressing EGFP, EGFP–NleB1 or EGFP–NleB1$_{AAA}$. UT, untransfected. Actin; loading control. Representative immunoblot from at least three independent experiments. **e,** Cleaved caspase-8 in HeLa cells infected with derivatives of EPEC and treated with FasL. Representative immunoblot from at least three independent experiments. pNleB1, pTrc99A carrying *nleB1*. pNleB2, pTrc99A carrying *nleB2*. **f,** Quantification of cleaved caspase-8 by immunofluorescence microscopy of HeLa cells infected with derivatives of EPEC and treated with FasL. Results are mean ± s.e.m. of the percentage of cells with cleaved caspase-8 from two independent experiments counting ~200 cells in triplicate. $^*P < 0.0001$ compared to uninfected, unstimulated control, one-way ANOVA. **g,** Immunofluorescence staining for detection of cleaved caspase-8 induced by FasL in HeLa cells infected with derivatives of EPEC. Scale bar, 10 μm. Representative images are shown from at least three independent experiments.

Transformation into a different yeast strain demonstrated that the interaction of NleB1 with FADD and RIPK1 occurred through their respective death domains (Fig. 1a–c). Studies in transfected HEK293T cells showed that FADD–Flag, TRADD–Flag and RIPK1–Flag co-purified with an EGFP–NleB1 (EGFP, enhanced green fluorescent protein) fusion protein as well as a 2HA-tagged NleB1 translocated during EPEC E2348/69 infection (Fig. 1d and Extended Data Fig. 1a, b). The death domain of FADD was required for binding to NleB1 from different attaching and effacing pathogens (Fig. 1d and Extended Data Fig. 1b). As TRADD and RIPK1 are critical components of the TNFR1 signalling complex, we investigated the influence of NleB1 on inhibition of the NF-κB dependent cytokine, IL-8, during EPEC infection. Unlike NleE, a known inhibitor of NF-κB activation[8,9], neither NleB1 nor its close homologue NleB2 appeared to inhibit IL-8 production during EPEC infection (Extended Data Fig. 1c). Therefore we focused our attention on FADD-dependent apoptosis signalling. After treatment with FasL, HeLa cells transfected with pEGFP–NleB1 showed significantly higher levels of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction (mitochondrial activity assay) compared to pEGFP transfected cells (Fig. 1e), suggesting that NleB1 blocked cell death. Immunoblot analysis with an antibody that recognizes only cleaved caspase-8, revealed that FasL-induced caspase-8 cleavage was substantially reduced in HeLa cells transfected with pEGFP–NleB1 (Fig. 1f). By immunofluorescence microscopy, fewer cells expressing EGFP–NleB1 contained cleaved caspase-8 after treatment with FasL (Extended Data Fig. 1d, e). In contrast, a comparable number of cells contained cleaved caspase-3 in untransfected, EGFP and EGFP–NleB1 expressing cells treated with tunicamycin (Extended Data Fig. 1e). This indicated that NleB1 had no effect on activation of the intrinsic apoptotic pathway.

NleB from the murine attaching and effacing pathogen *C. rodentium* was recently described as an *N*-acetylglucosamine (GlcNAc) transferase and a member of the glycogenin family of enzymes[10]. Given the ability of NleB1 to bind FADD and inhibit proteolytic activation of caspase-8, we examined whether FADD was post-translationally modified by NleB1. Following incubation with GST–NleB1 and UDP-GlcNAc, we observed GlcNAc modification of His–FADD (Fig. 2a). This modification was not present upon incubation with an NleB1 catalytic site mutant (NleB1$_{AAA}$)[10]. Similar modification of FADD–Flag occurred upon ectopic expression of EGFP–NleB1 in HeLa cells (Extended Data Fig. 1f). Intact protein liquid chromatography–mass spectrometry (LC–MS) of His–FADD incubated with GST–NleB1 and UDP-GlcNAc revealed a mass shift matching a single GlcNAc modification on FADD (Fig. 2b). Peptide sequencing of multiple spectra

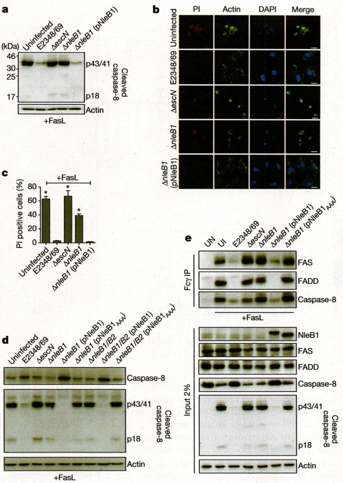

Figure 3 | Inhibition of FasL-induced DISC formation and cell death by EPEC. **a**, Cleaved caspase-8 in HeLa cells infected with derivatives of EPEC. Actin; loading control. Representative immunoblot from at least three independent experiments. **b**, Cell death visualized by propidium iodide (PI) staining in HeLa cells infected with derivatives of EPEC and treated with FasL. Scale bar, 20 μm. Representative images shown from at least three independent experiments. **c**, Quantification of PI staining and microscopic analysis in HeLa cells infected with derivatives of EPEC and treated with FasL. Results are mean ± s.e.m. of percentage cells with PI staining from two independent experiments counting ~200 cells in triplicate. $^{*}P < 0.0001$ compared to E2348/69 infected cells, one-way ANOVA. **d**, Cleaved and full-length caspase-8 in HeLa cells infected with derivatives of EPEC and treated with FasL. Representative immunoblot from at least three independent experiments. **e**, DISC components induced by FasL coupled to Fcγ and precipitated with protein G beads. UN, untreated and uninfected; UI, uninfected. Representative immunoblot from at least three independent experiments.

from in-gel digests unambiguously identified Arg 117 as the site of *N*-acetylglucosylation (Fig. 2c, Extended Data Figs 2–3, Supplementary Table 1). This was confirmed by substitution of Arg 117 in FADD with alanine, whereas alanine substitution at Ser 122 had no effect on NleB-mediated *N*-acetylglucosylation (Extended Data Fig. 4). Arg 117 is located at the interface of the FAS–FADD death domain interaction and is critical for assembly of the FAS–FADD oligomeric complex and formation of the DISC[11,12]. Accordingly, EGFP–NleB1 but not catalytically inactive EGFP–$\mathrm{NleB1_{AAA}}$ inhibited caspase-8 activation (Fig. 2d).

During infection, NleB1 delivered by the EPEC T3SS inhibited FasL-induced caspase-8 activation in HeLa cells. This inhibition was lost in cells infected with an Δ*escN* mutant that has a non-functional T3SS, or an Δ*nleB1*/*B2* double mutant (Fig. 2e–g). Complementation of the Δ*nleB1*/*B2* mutant with *nleB1* but not *nleB2* restored the ability of EPEC to inhibit caspase-8 activation (Fig. 2e–g), demonstrating that the two NleB proteins have distinct functions. This mirrored our protein interaction studies, which showed that NleB2 did not interact with FADD or TRADD and bound only weakly to RIPK1 (Extended Data Fig. 5a–d).

Arg 117 in FADD is essential for FAS–FADD and TRADD–FADD death domain interactions[11,12]. Consistent with the role of NleB1 in inhibition of FADD-dependent caspase-8 activation, an *nleB1* mutant was unable to inhibit FasL- or TNF-induced activation of caspase-8 whereas the complemented strain was as effective as wild-type EPEC (Fig. 3a and Extended Data Fig. 5e, f). EPEC expressing NleB1 also inhibited FasL-induced apoptosis as measured by propidium iodide (PI) staining (Fig. 3b, c). These phenotypes were not due to differences in adherence (Extended Data Fig. 5g).

NleB1 *N*-acetylglucosamine transferase activity was required for inhibition of FasL-induced caspase-8 processing during infection (Fig. 3d). To examine the influence of NleB1 on FAS DISC assembly, which precedes caspase-8 activation, we immunoprecipitated the FAS receptor complex from infected HeLa cells treated with FasL (Fig. 3e). Prior infection of cells with EPEC substantially inhibited FasL-induced DISC formation and this required NleB1 *N*-acetylglucosamine transferase activity (Fig. 3e).

We next examined the effect of NleB on inhibition of caspase-8 processing *in vivo* by infecting wild-type C57BL/6 mice with *C. rodentium* or a Δ*nleB* mutant of *C. rodentium* and staining tissue sections for cleaved caspase-8. Although few cells were positive for cleaved caspase-8 during *C. rodentium* infection, significant numbers of cells were positive for cleaved caspase-8 during infection with the Δ*nleB* mutant (Fig. 4a). Many of these caspase-8 positive cells were present as sloughed cells in the gut lumen (Extended Data Fig. 6a).

The observation that NleB1 inhibited FasL-induced caspase-8 cleavage suggested that FAS-mediated apoptosis contributes to host defence against attaching and effacing pathogens. We tested this by infecting FAS-deficient $Fas^{lpr/lpr}$ mutant mice with *C. rodentium*[13]. As shown previously, *nleB* was essential for efficient colonization of wild-type C57BL/6 mice[14] (Fig. 4b). Although there was no difference in faecal shedding of *C. rodentium* between C57BL/6 and $Fas^{lpr/lpr}$ mice during the acute phase of infection (first 12 days) (Fig. 4b), $Fas^{lpr/lpr}$ mice developed severe, watery diarrhoea (Fig. 4c). In addition, *C. rodentium* penetrated the crypts in $Fas^{lpr/lpr}$ mice, unlike in C57BL/6 mice in which the bacteria were predominantly located at the tips of the villi (Fig. 4d). To study the resolving phase of infection (post day 12), C57BL/6 and $Fas^{lpr/lpr}$ mice were infected with a lower dose of *C. rodentium* to avoid the rapid onset of severe diarrhoea and monitored for up to 33 days. $Fas^{lpr/lpr}$ mice were impaired in their ability to clear *C. rodentium* compared to C57BL/6 mice (Fig. 4e and Extended Data Fig. 6b).

The development of severe diarrhoea in *C. rodentium*-infected $Fas^{lpr/lpr}$ mice was unexpected and sections of colon from mice infected with *C. rodentium* for 10 days were examined to identify differences in pathology between $Fas^{lpr/lpr}$ mice and C57BL/6 mice. Histological analysis of colons from $Fas^{lpr/lpr}$ mice revealed greater erosion of the epithelium (tissue damage) as well as increased colon weight and crypt height compared to C57BL/6 mice (measures of *C. rodentium* induced intestinal hyperplasia[14]) (Extended Data Fig. 7a–d). In addition, $Fas^{lpr/lpr}$ mice showed more pronounced neutrophil infiltration, which penetrated across the muscularis mucosa (Extended Data Fig. 7e). Similar phenotypes were observed in FasL-deficient $Fas^{gld/gld}$ mice and Bid-deficient mice (Fig. 4d, e and Extended Data Fig. 8). Bid is not required for FasL-induced apoptosis in lymphoid cells, but is required for FasL-induced apoptosis in several other cell types[15,16]. This suggests that FAS signalling in epithelial cells rather than lymphoid cells limits *C. rodentium*-induced colitis.

We reasoned that if NleB interfered with FAS-mediated apoptosis signalling during infection, then *C. rodentium* Δ*nleB* mutant bacteria would no longer be attenuated in mice that carry defects in this apoptosis pathway. Indeed, $Fas^{lpr/lpr}$ mice infected with the Δ*nleB* mutant showed comparable levels of bacterial colonization and severity of diarrhoea compared to their littermates infected with *C. rodentium* (Extended

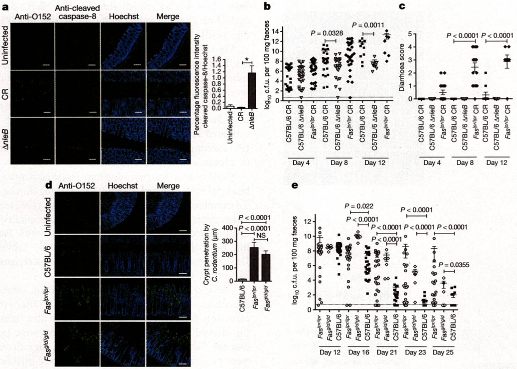

Figure 4 | Infection of mice deficient for FAS signalling with *C. rodentium*. **a**, Immunofluorescence staining and quantification of cleaved caspase-8 in colonic sections from C57BL/6 mice infected with *C. rodentium* (CR) or a *C. rodentium nleB* mutant. Representative images from at least three separate sections of colon at least 100 μm apart (transverse or longitudinal), per animal from five individual mice per group. Results are mean ± s.e.m. of percentage fluorescence intensity of cleaved caspase-8 staining relative to Hoechst staining of five fields with *C. rodentium* infection from three mice per group. $*P = 0.0001$, unpaired two-tailed *t*-test. **b**, Bacterial load in the faeces during acute infection with *C. rodentium*. Each data point represents $\log_{10}$ c.f.u. per 100 mg faeces per individual animal on days 4, 8 and 12 after infection (c.f.u., colony forming units). Mean ± s.e.m. are indicated, dotted line represents the detection limit. *P* values from Mann–Whitney *U*-test. **c**, Diarrhoea score at day 4, 8 and 12 post-infection. The scoring system is described in the Methods. Mean ± s.e.m. are indicated. *P* values from one-way ANOVA. **d**, Immunofluorescence staining and quantification of *C. rodentium* penetration into intestinal crypts in colonic sections from C57BL/6, $Fas^{lpr/lpr}$ and $Fas^{gld/gld}$ mice. Representative images from at least two separate sections of colon at least 100 μm apart (transverse or longitudinal), per animal from five individual mice per group. Results are the mean ± s.e.m. maximum distance of *C. rodentium* staining from the epithelial surface (in μm) of five independent sections with at least three measurements per section. $P = 0.0001$, unpaired two-tailed *t*-test. **e**, Bacterial load in the faeces during the resolving phase of *C. rodentium* infection. Each data point represents $\log_{10}$ c.f.u. per 100 mg faeces per individual animal on days 12, 16, 21, 23 and 25 post-infection. Mean ± s.e.m. are indicated, dotted line represents detection limit. *P* values from Mann–Whitney *U*-test. Scale bars, 100 μm.

Data Figs 6b and 9a, b). The same was not true for Δ*espI* or Δ*espF* mutants of *C. rodentium*, which retained their known colonization defects in $Fas^{lpr/lpr}$ mice (Extended Data Fig. 9c, d)[17,18]. EspI and EspF are T3SS effector proteins with functions unrelated to NleB1 and the inhibition of FAS signalling.

During infection with attaching and effacing pathogens, the predominant site of NleB activity is in enterocytes where the translocation of bacterial effector proteins occurs[1]. NleB-mediated inhibition of FasL/FAS-induced apoptosis may prolong bacterial attachment to host cells and thereby promote bacterial shedding in the faeces. Indeed NleB is associated with the transmission of *C. rodentium* amongst littermates[19] and is epidemiologically associated with outbreaks of haemolytic uraemic syndrome caused by EHEC[20,21]. Conversely, the reduced colonization of C57BL/6 mice by *C. rodentium* Δ*nleB* mutants and increased numbers of activated caspase-8 positive cells may reflect partial protection by the FAS apoptotic pathway through the elimination of infected cells[14]. This is consistent with the prolonged shedding of *C. rodentium* in FAS-deficient animals compared to C57BL/6 mice. The increased intestinal pathology observed in *C. rodentium*-infected FAS-, FasL- and Bid-deficient mice has also revealed a function for the FAS apoptotic pathway in limiting the extent of colitis during gastrointestinal infection. This aggravation of disease may be mirrored in humans, as nucleotide polymorphisms in the *FASLG* gene, encoding FasL, have been associated with the development of inflammatory bowel disease[22]. In summary, the discovery of a bacterial T3SS effector that specifically modifies death domain containing proteins through a newly identified post-translational modification and thereby inhibits death receptor mediated apoptosis has revealed a new virulence mechanism in bacterial infection.

METHODS SUMMARY

Detailed information including bacterial mutant construction, immunofluorescence, immunoblot details, mouse infections, histology and pathology scoring, imaging and image analysis, and associated references are included in the Methods. Protein interaction assays were investigated using the yeast two-hybrid system and co-immunoprecipitation. Activation of caspase-8 was assessed by immunoblot and immunofluorescence microscopy using antibodies specific for cleaved caspase-8. Cell death was examined by MTT reduction and propidum iodide staining. Post-translational modifications of FADD were identified by intact protein mass spectrometry and collision-induced dissociation (CID) and electron transfer dissociation (ETD) peptide fragmentation. All genetically modified and spontaneous mutant mice are on a C57BL/6 background and all animals were inoculated by oral gavage with 200 μl containing approximately 1×10^9 or 1×10^8 c.f.u. of *C. rodentium*. All animal experimentation was approved by the University of Melbourne Animal Ethics Committee, applications 0808209 and 1112062.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions J.S.P., C.G., S.Y.O., C.L.K., M.K., K.S.R., T.W.F.L., P.R., C.V.O. and A.B. designed and performed the experiments. V.F.C. and O.M. generated reagents. C.S.A., N.A.W., G.I. and A.I.W. performed mass spectrometry analyses. L.A.O., A.Z., S.M., U.N., A.M., A.S., J.S., G.F., J.S.P. and E.L.H. contributed to experimental design. J.S.P., A.S., J.S., G.F. and E.L.H. wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to E.L.H. (hartland@unimelb.edu.au).

LETTER

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Stability and function of regulatory T cells is maintained by a neuropilin-1–semaphorin-4a axis

Greg M. Delgoffe[1]*, Seng-Ryong Woo[1]*, Meghan E. Turnis[1], David M. Gravano[1], Cliff Guy[1], Abigail E. Overacre[1,2], Matthew L. Bettini[1], Peter Vogel[3], David Finkelstein[4], Jody Bonnevier[5], Creg J. Workman[1] & Dario A. A. Vignali[1]

Regulatory T cells (T_{reg} cells) have a crucial role in the immune system by preventing autoimmunity, limiting immunopathology, and maintaining immune homeostasis[1]. However, they also represent a major barrier to effective anti-tumour immunity and sterilizing immunity to chronic viral infections[1]. The transcription factor Foxp3 has a major role in the development and programming of T_{reg} cells[2,3]. The relative stability of T_{reg} cells at inflammatory disease sites has been a highly contentious subject[4–6]. There is considerable interest in identifying pathways that control the stability of T_{reg} cells as many immune-mediated diseases are characterized by either exacerbated or limited T_{reg}-cell function. Here we show that the immune-cell-expressed ligand semaphorin-4a (Sema4a) and the T_{reg}-cell-expressed receptor neuropilin-1 (Nrp1) interact both *in vitro*, to potentiate T_{reg}-cell function and survival, and *in vivo*, at inflammatory sites. Using mice with a T_{reg}-cell-restricted deletion of Nrp1, we show that Nrp1 is dispensable for suppression of autoimmunity and maintenance of immune homeostasis, but is required by T_{reg} cells to limit anti-tumour immune responses and to cure established inflammatory colitis. Sema4a ligation of Nrp1 restrained Akt phosphorylation cellularly and at the immunologic synapse by phosphatase and tensin homologue (PTEN), which increased nuclear localization of the transcription factor Foxo3a. The Nrp1-induced transcriptome promoted T_{reg}-cell stability by enhancing quiescence and survival factors while inhibiting programs that promote differentiation. Importantly, this Nrp1-dependent molecular program is evident in intra-tumoral T_{reg} cells. Our data support a model in which T_{reg}-cell stability can be subverted in certain inflammatory sites, but is maintained by a Sema4a–Nrp1 axis, highlighting this pathway as a potential therapeutic target that could limit T_{reg}-cell-mediated tumour-induced tolerance without inducing autoimmunity.

It was suggested initially that T_{reg}-cell-mediated suppression was contact-dependent, and soluble factors were insufficient, as purified T_{reg} cells failed to suppress across a permeable transwell membrane[3,7]. However, we have shown previously that additional signals, derived from co-cultured T cells, are required to potentiate maximal T_{reg}-cell suppression by soluble factors across a transwell membrane[8]. Indeed, T_{reg} cells stimulated in the presence of fixed or live $CD4^+$ conventional T cells (T_{conv} cells) in the top chamber of a transwell plate can suppress T_{conv} cells in the bottom chamber (Supplementary Fig. 1a). To determine the T_{conv}-cell-derived signals responsible for potentiating or boosting T_{reg}-cell-mediated suppression, we compared the T_{conv}- and T_{reg}-cell transcriptomes and identified Sema4a as a potential ligand that could boost T_{reg}-cell function (Supplementary Fig. 1b–d). Sema4a is involved in neural patterning and has been implicated in modulating immune function, with haematopoietic cell expression restricted to $CD4^+$ and $CD8^+$ T cells, natural killer cells and dendritic cells[9]. Using RNA interference, loss-variant selection and overexpression in hybridomas, and

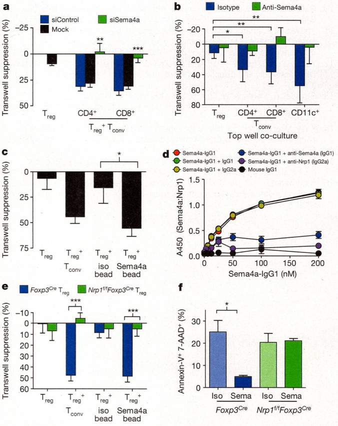

Figure 1 | Sema4a binds Nrp1 to potentiate T_{reg}-cell function and survival *in vitro*. a, Transwell suppression assay in which T_{reg} cells were co-cultured in the top chamber of a transwell plate with anti-CD3- and anti-CD28-coated beads in the presence or absence of $CD4^+$ or $CD8^+$ T_{conv} cells that had been transfected previously with scrambled or siRNA to *Sema4a*. Proliferation of T_{conv} cells stimulated with anti-CD3- and anti-CD28-coated beads in the bottom chambers was measured by [^{3}H]-thymidine uptake. **b,** Transwell suppression assay with T_{reg} cells co-cultured in top chamber with $CD4^+$, $CD8^+$ or $CD11c^+$ cells including anti-Sema4a or its isotype control. **c,** Transwell suppression assay in which T_{reg} cells were co-cultured in the absence of T_{conv} cells but in the presence of beads coated with Sema4a-IgG1 or its isotype control. **d,** ELISA-based binding assay in which plates coated with Nrp1 were incubated with Sema4a-IgG1 or its isotype control in the presence of various blocking antibodies. Sema4a-IgG1 was detected using an isotype specific antibody. **e,** Transwell suppression assay in which T_{reg} cells were purified by flow cytometry from *$Foxp3^{Cre}$* or *$Nrp1^{f/f}Foxp3^{Cre}$* mice. **f,** Annexin V-7-AAD staining of T_{reg} cells stimulated for 48 h *in vitro* in the presence of Sema4a-IgG1 or its isotype control. Results represent the mean of five independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired *t*-test. Error bars indicate s.e.m.

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[1]Department of Immunology, St Jude Children's Research Hospital, Memphis, Tennessee 38105, USA. [2]Integrated Biomedical Sciences Program, University of Tennessee Health Science Center, Memphis, Tennessee 38163, USA. [3]Department of Pathology, St Jude Children's Research Hospital, Memphis, Tennessee 38105, USA. [4]Computational Biology, St Jude Children's Research Hospital, Memphis, Tennessee 38105, USA. [5]R&D Systems Inc., Minneapolis, Minnesota 55413, USA.

*These authors contributed equally to this work.

antibody blockade, we confirmed that Sema4a was necessary for T_{conv} and dendritic cells to potentiate T_{reg}-cell-mediated transwell suppression (Fig. 1a, b and Supplementary Fig. 2a–e). Sema4a is also sufficient to potentiate suppression, as T_{reg} cells cultured in the presence of beads coated with a Sema4a-immunoglobulin-G1 (IgG1) fusion protein, instead of fixed T_{conv} cells, potentiated transwell suppression (Fig. 1c). In contrast, soluble Sema4a-IgG1 inhibited T_{conv}-cell-potentiated, T_{reg}-cell-mediated transwell suppression (Supplementary Fig. 2f). These data demonstrate that Sema4a is required and sufficient to potentiate T_{reg}-cell function by soluble factors *in vitro*.

Nrp1 is a homogeneously expressed marker of thymically derived T_{reg} cells, and has also been shown to bind Sema3a, which shares homology with Sema4a, and vascular endothelial growth factor (VEGF) in mediating neural axon growth and angiogenesis (Supplementary Fig. 3a)[10–13]. We therefore proposed that Nrp1 functions as a receptor for Sema4a on T_{reg} cells. Sema4a could directly and specifically bind to Nrp1, albeit with slightly lower avidity to Sema3a, as demonstrated in an enzyme-linked immunosorbant assay (ELISA)-based binding assay with recombinant Nrp1 and Sema family proteins and blocking antibodies to Nrp1 or Sema4a (Fig. 1d and Supplementary Fig. 3b–d). We bred mice harbouring a loxP-flanked ('floxed') *Nrp1* gene (*Nrp1*$^{f/f}$) to mice expressing a yellow fluorescent protein (YFP)-codon-improved Cre (iCre) fusion protein driven by an IRES following *Foxp3* (*Foxp3*$^{YFP-iCre}$, which we refer to as *Foxp3*^{Cre}) to obtain a T_{reg}-cell-restricted deletion of Nrp1 (*Foxp3*$^{YFP-iCre}$ × *Nrp1*$^{f/f}$, which we refer to as *Nrp1*$^{f/f}$*Foxp3*^{Cre}). Sema4a-IgG1 also bound to control but not Nrp1-deficient T_{reg} cells in a flow cytometric assay (Supplementary Fig. 3e). These data support a direct interaction between Nrp1 and Sema4a.

Although Nrp1-deficient T_{reg} cells develop normally and can suppress in a 'classical' contact-dependent suppression assay (Supplementary Fig. 4a), they were unable to suppress across a transwell membrane in the presence of either T_{conv} cells or Sema4a-IgG1-coated beads (Fig. 1e). Transwell suppression can be blocked by anti-Nrp1 and, as reported previously, is dependent on interleukin-10 (IL-10) and IL-35 (Supplementary Fig. 4b–d)[8]. Sema4a–Nrp1 ligation substantially reduced cell death and modestly increased proliferation, and, although single-cell levels of IL-10 and IL-35 did not change, increased survival resulted in greater amounts of IL-10 and IL-35 in Sema4a-treated T_{reg}-cell cultures (Fig. 1f and Supplementary Fig. 4e–i). Expression of NRP1 on human T_{reg} cells has been a contentious subject[14,15]. We observed sustained NRP1 expression, albeit modest, on optimally suppressive T_{reg} cells that could mediate transwell suppression in response to human SEMA4A in a NRP1-dependent manner (Supplementary Fig. 5). Taken together, these data suggest Sema4a–Nrp1 interaction promotes T_{reg}-cell survival and function.

Nrp1$^{f/f}$*Foxp3*^{Cre} mice did not show any autoimmune phenotype for at least 16 months after birth (data not shown). In addition, the capacity of *Foxp3*^{Cre} and *Nrp1*$^{f/f}$*Foxp3*^{Cre} T_{reg} cells to limit the development of the autoimmune sequelae caused by *Foxp3* deletion was comparable (Supplementary Fig. 6), suggesting that maintenance of immune homeostasis and prevention of autoimmunity may not require Nrp1 signalling.

We reasoned that Nrp1 signalling may regulate T_{reg}-cell function under inflammatory conditions. T_{reg} cells are recruited to and are induced by tumour cells, which consequently hamper protective anti-tumour immunity[16]. *Foxp3*$^{DTR-GFP}$ mice, which allow for conditional T_{reg}-cell deletion following diphtheria toxin treatment[17], can clear MC38 adenocarcinoma, EL4 thymoma, and B16 melanoma tumours when treated with diphtheria toxin at the time of inoculation, although they invariably succumb to autoimmune disease (Fig. 2a–c). Notably, *Nrp1*$^{f/f}$*Foxp3*^{Cre} mice showed reduced, delayed tumour growth and increased survival,

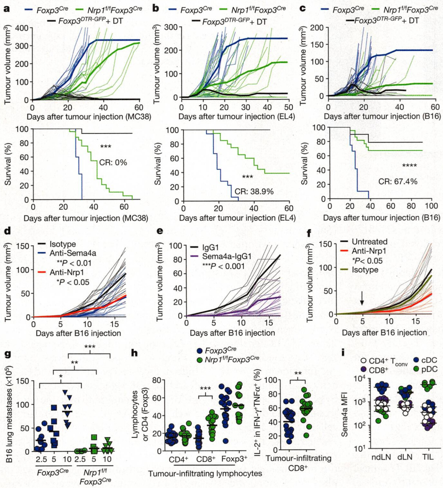

Figure 2 | Nrp1-deficient T_{reg} cells fail to suppress anti-tumour immune responses. **a**, Tumour growth curve (top) and survival plot (bottom) of *Foxp3*^{Cre}, *Nrp1*$^{f/f}$*Foxp3*^{Cre}, or *Foxp3*$^{DTR-GFP}$ mice receiving 1.25 × 10^5 MC38 melanoma cells subcutaneously and (for *Foxp3*$^{DTR-GFP}$) 100 μg diphtheria toxin (DT) intraperitoneally, twice weekly. **b**, As in **a**, but mice received 1.25 × 10^5 EL4 thymoma intradermally. **c**, As in **a**, but mice received 1.25 × 10^5 B16 melanoma intradermally. **d**, Tumour growth curve of C57/BL6 mice receiving 1.25 × 10^5 B16 melanoma intradermally concomitant with injections of isotype control, anti-Sema4a, or anti-Nrp1 (100 μg) twice weekly. **e**, Tumour growth curve as in **d** except mice received Sema4a-IgG1 twice weekly. **f**, Tumour growth curve of C57/BL6 mice receiving 1.25 × 10^5 B16 melanoma intradermally. When tumours were palpable (day 5, indicated by arrow), mice began receiving injections of anti-Nrp1 or its isotype control (400 μg initially, 200 μg every 3 days). **g**, Lung metastasis counts from *Foxp3*^{Cre} or *Nrp1*$^{f/f}$*Foxp3*^{Cre} mice injected with 2.5–10 × 10^5 B16 cells intravenously, 17–20 days earlier. **h**, Tabulation of flow cytometric analysis of tumour-infiltrating lymphocytes from *Foxp3*^{Cre} or *Nrp1*$^{f/f}$*Foxp3*^{Cre} mice injected intradermally with B16 18 days earlier. **i**, Sema4a expression of various immune cells in nondraining lymph node (ndLN), draining lymph node (dLN) or tumour-infiltrating lymphocytes (TIL). Results represent the mean of five (**a–c**, n = 10–25 mice), three (**d–h**, n = 8–20 mice), or three (**i**) experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, by (**a–f**) one-way analysis of variance (ANOVA) or (**g–i**) unpaired t-test. CR, complete response. Error bars indicate s.e.m.

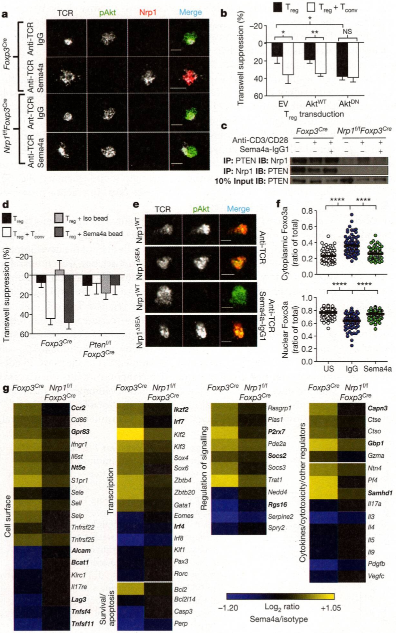

Figure 3 | Ligation of Nrp1 by Sema4a promotes T_{reg}-cell stability through modulation of Akt–mTOR signalling. a, Total internal reflection fluorescence (TIRF) microscopic analysis of immunologic synapse Akt phosphorylation in T_{reg} cells stimulated 20 min on a lipid bilayer coated with anti-TCR antibodies in the presence or absence of Sema4a-IgG1. **b**, Transwell suppression assay using T_{reg} cells retrovirally expressing wild-type (WT) or dominant-negative (DN)-Akt. Transductants were selected and expanded using puromycin and IL-2. NS, not significant. **c**, Immunoprecipitation (IP) analysis of Nrp1 using *in vitro* expanded T_{reg} cells that were serum starved for 3 h, then stimulated as indicated for 6 h before IP. **d**, Transwell suppression assay using *Foxp3*^{Cre} or *Pten*$^{f/f}$*Foxp3*^{Cre} T_{reg} cells. **e**, TIRF microscopic analysis of immunologic synapse Akt phosphorylation as in **a**, of *Nrp1*$^{f/f}$*Foxp3*^{Cre} T_{reg} cells retrovirally reconstituted with wild-type (WT) or SEA-deficient (ΔSEA) Nrp1. **f**, Foxo3a cytoplasmic (top) and nuclear (bottom) localization signals, as defined by masking using actin and DNA staining. Arbitrary Units represent fluorescence intensity calculated volumetrically through 20 to 30 slices of T_{reg} cells. $n = 70$–93. US, unstimulated (control). **g**, Heat map (right) of genes regulated by Nrp1. *Foxp3*^{Cre} and *Nrp1*$^{f/f}$*Foxp3*^{Cre} CD45RB^{lo} Foxp3 (YFP)$^{+}$ CD4^{+} T cells were stimulated for 48 h with anti-CD3, anti-CD28, 100 units per ml rhIL-2, and immobilized IgG1 or Sema4a-IgG1. RNA was subjected to Affymetrix gene profiling. T_{reg}-cell signature genes are in bold. All genes shown met false discovery rate (FDR) <0.10 and compared using two-way ANOVA. Results represent at least three independent experiments (**a**, **c**, **e**, **f**) or represent means of three (**b**, **d**) or seven (**g**) experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ by unpaired *t*-test. Error bars indicate s.e.m.

particularly with B16 melanoma, without any detectable autoimmune consequences (Fig. 2a–c and data not shown). In wild-type C57BL/6 mice, blockade of this pathway using Sema4a monoclonal antibody, Nrp1 monoclonal antibody (which does not block Nrp1–VEGF interaction), and Sema4a-IgG1 (which acts as a soluble antagonist), significantly decreased tumour growth (Fig. 2d–f and Supplementary Fig. 2f, 3b, c). Nrp1-deficient T_{reg} cells also failed to suppress clearance of B16 lung metastases, even with very high tumour cell inoculates (Fig. 2g and Supplementary Fig. 7a). *Nrp1*$^{f/f}$*Foxp3*^{Cre} mice displayed increased intratumoral CD8^{+} T cells, particularly in the IFN-γ^{+}IL-2^{+}TNF-α^{+} tumoricidal subset (Fig. 2h)[18]. Although we originally presumed the source of Sema4a would be tumour-infiltrating T cells and conventional dendritic cells, the majority of Sema4a^{+} cells infiltrating tumours were plasmacytoid dendritic cells (57.4% of intratumoral Sema4a^{+} cells), consistent with previous suggestions highlighting mechanistic links between T_{reg} cells and plasmacytoid dendritic cells in mediating tumour-induced tolerance (Fig. 2i and Supplementary Fig. 7b, c)[19]. Indeed, plasmacytoid dendritic cells can potentiate T_{reg}-cell function *in vitro* in a Sema4a–Nrp1-dependent manner (Supplementary Fig. 7d). Treatment with Sema4a-IgG1 also resulted in an increased number of intratumoral CD8^{+} T cells, consistent with observations with *Nrp1*$^{f/f}$*Foxp3*^{Cre} mice (Supplementary Fig. 7e). Nrp1-deficient T_{reg} cells also fail to cure established inflammatory colitis, suggesting that the utilization of this pathway is not restricted to the tumour microenvironment (Supplementary Fig. 8). Thus, although Nrp1 seems to be dispensable for regulating immune homeostasis, it is required for maximal T_{reg}-cell-mediated control of inflammatory environments.

We next sought to determine the signalling pathway downstream of Nrp1. Given the importance of limiting Akt–serine/threonine-protein kinase mTOR signalling in T_{reg}-cell function, and previous suggestions that Nrp1 modulates Akt signalling, we proposed that Nrp1 inhibits Akt function in T_{reg} cells[20–22]. Indeed, T-cell receptor (TCR)- and CD28-activation-induced whole-cell Akt–mTOR signalling (as determined by phosphorylation of Akt and S6K1) was reduced to baseline levels in freshly isolated T_{reg} cells (as well as 47–54% in IL-2-expanded T_{reg} cells) by Sema4a-mediated Nrp1 ligation (Supplementary Fig. 9a, b). Furthermore, T_{reg}-cell activation on stimulatory lipid bilayers containing Sema4a-IgG1, but not an isotype control, recruited Nrp1 to the immunologic synapse and inhibited immunologic synapse Akt phosphorylation, while sparing global immunologic synapse tyrosine

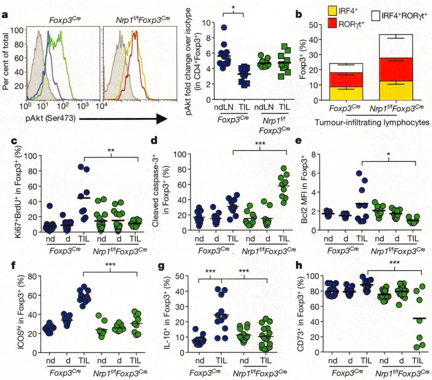

Figure 4 | Tumour-infiltrating T_{reg} cells bear a signature similar to Sema4a–Nrp1 ligation. **a**, Akt phosphorylation in T_{reg} cells. TIL (blue and maroon) and ndLN (green and gold) were collected from tumour-bearing $Foxp3^{Cre}$ or $Nrp1^{f/f}Foxp3^{Cre}$ mice. Cells were immediately fixed and stained for phosphorylated Akt (pAkt). **b–h**, Shaded histogram indicates isotype control. Results are tabulated normalized to isotype control staining. IRF4 and RORγT (**b**), Ki67 and BrdU (**c**), cleaved caspase-3 (**d**), Bcl2 (**e**), ICOS (**f**), IL-10 (**g**) and CD73 (**h**) staining from ndLN, dLN, or TIL from tumour-bearing $Foxp3^{Cre}$ or $Nrp1^{f/f}Foxp3^{Cre}$ mice. Ki67 and BrdU analysis included injection with BrdU 14 h before harvest. IL-10 staining included restimulation with phorbol myristate acetate (PMA) and ionomycin for 16 h in the presence of brefeldin A. Results represent the mean of 3–5 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by paired *t*-test (**a**, $n = 10$–11) or unpaired *t*-test (**b–h** $n = 7$–17). MFI, mean fluorescence intensity. Error bars indicate s.e.m.

phosphorylation (Fig. 3a and Supplementary Fig. 9c, d). Retroviral overexpression of a dominant-negative Akt mutant[23] in T_{reg} cells expanded with IL-2 limited the requirement for Nrp1 ligation, at least in transwell suppression assays, suggesting that Akt may be a dominant pathway that limits T_{reg}-cell transwell suppression (Fig. 3b and Supplementary Fig. 9e). As immunologic synapse phosphorylated-Akt diminution in response to Sema4a was rapid, we reasoned that Nrp1 recruits the phosphatase PTEN to the immunologic synapse, restraining Akt–mTOR signalling[24]. Nrp1 constitutively bound PTEN in resting T_{reg} cells, which was reduced upon activation but maintained in the presence of Sema4a–Nrp1 interaction (Fig. 3c). PTEN-deficient T_{reg} cells failed to inhibit Akt phosphorylation at the immunologic synapse in the presence of Sema4a, and failed to suppress across a transwell membrane in response to Sema4a- or T_{conv}-cell-mediated potentiation (Fig. 3d and Supplementary Fig. 10a, b). Nrp1 has a small cytoplasmic domain consisting of an evolutionarily conserved PDZ domain-binding motif (carboxy-terminal amino acid sequence: Ser-Glu-Ala)[25]. Nrp1 mutants lacking this motif could not inhibit Akt phosphorylation at the immunologic synapse or recruit PTEN (Fig. 3e and Supplementary Fig. 10c, d). Activated Akt can phosphorylate Foxo transcription factors, promoting their exclusion from the nucleus[26]. Foxo transcription factors are critical for T_{reg}-cell development, through interaction with Foxp3 as well as inducing several T_{reg}-cell signature genes[26–28]. Indeed, Sema4a-IgG1 limited the activation-induced nuclear export of Foxo3a (Fig. 3f). We propose that, during activation, Nrp1 ligation restrains Akt phosphorylation through PTEN, facilitates Foxo nuclear localization, and thereby potentiates T_{reg}-cell function.

Gene-expression analysis revealed an Nrp1-induced transcriptional profile that was consistent with promoting T_{reg}-cell survival, stability and quiescence, and that was similar to a Foxo-dependent transcriptional signature (Fig. 3g)[26–28]. Gene Ontology and Gene Set Enrichment Analysis revealed that Nrp1 ligation modulated multiple pathways and programs, including the IL-2- and IL-7-related transcriptional programs, repression of cytokine transcripts and modulated Foxp3 targets (Fig. 3g, Supplementary Fig. 11–13, and Supplementary Tables 1 and 2). Of particular interest was the stabilization of the transcription factor Kruppel-like Factor 2 (KLF2) (and its targets *Sell, Ccr7, Il7r*), the T_{reg}-cell regulator Helios (*Ikzf2*; also known as IKAROS family zinc finger 2), and the anti-apoptotic protein Bcl2, accompanied by a concomitant repression of the lineage defining transcription factors Eomes, IRF4 and RORγt (nuclear receptor ROR-γt, encoded by *Rorc*), suggesting a role for Nrp1 in stabilizing the T_{reg}-cell program and repressing terminal differentiation (Fig. 3g and Supplementary Fig. 13b–d).

Finally, we sought to determine whether the molecular fingerprints of Nrp1 signalling could be observed *in vivo*. Tumour-infiltrating T_{reg}-cells restrained Akt phosphorylation in an Nrp1-dependent manner (Fig. 4a). Likewise, tumour-infiltrating T_{reg} cells showed Nrp1-dependent upregulation of Helios and downregulation of IRF4 and RORγt (Fig. 4b and Supplementary Fig. 14a, b). This was associated with an increase in intratumoral T_{reg}-cell proliferation, as revealed by Ki67-BrdU (5-bromodeoxyuridine) staining, reduced caspase-3-dependent programmed cell death, and increased expression of the anti-apoptotic protein Bcl2 (Fig. 4c–e and Supplementary Fig. 14c–e). We also observed an Nrp1-dependent increase in the percentage of ICOS$^+$, IL-10$^+$ and CD73$^+$ intratumoral T_{reg} cells (Fig. 4f–h and Supplementary Fig. 14f–h). Taken together, our data support a role for Nrp1 in modulating T_{reg}-cell stability, survival and function in certain tumour microenvironments.

Our data demonstrate that cell contact-dependent potentiation of T_{reg}-cell stability and function is mediated by Sema4a–Nrp1 ligation through a PTEN–Akt–Foxo axis (Supplementary Fig. 15). This pathway enhances T_{reg}-cell function indirectly by enforcing stability and promoting survival, and this is most evident in inflammatory sites such as certain tumours and colitic intestinal mucosa. However, Nrp1 signalling may also boost T_{reg}-cell function directly by enhancing some suppressive mechanisms (for example, CD73). Apart from haematopoietic lineages, Sema4a seems to have a pattern of expression consistent with sites in which T_{reg}-cell tolerogenic activity would be desired, such as the nervous system, eye and intestine[29]. The issue of T_{reg}-cell stability has been highly contentious, and the mechanisms that maintain T_{reg}-cell stability remain elusive[4–6]. As Foxo family members enhance Foxp3 function and promote T_{reg}-cell homeostasis and function[26–28], it is intriguing that Nrp1 signalling may counteract the negative impact of Akt on Foxo nuclear localization, resulting in substantial overlap between the transcriptional profiles induced by Foxo and Nrp1 signalling[28]. It is possible that the Nrp1–Sema4a pathway may be perturbed genetically or under certain pathologic circumstances; this could also provide a basis for the seemingly contradictory perceptions of T_{reg}-cell stability in a variety of normal and diseased states.

Previous studies have shown that plasmacytoid dendritic cells promote tolerance, T_{reg}-cell differentiation and function, and their ablation has been shown to correlate with enhanced antitumour immunity[19,30]. Given that the dominant intratumoral source of Sema4a was plasmacytoid dendritic cells, this raises the possibility that Nrp1-induced T_{reg}-cell stability and survival provides a mechanistic explanation for these observations. As T_{reg} cells represent a major barrier to anti-tumour immunity in many cancers, a clinically relevant and critical question is whether it is possible to limit T_{reg}-cell function in tumours while preventing inflammatory or autoimmune adverse events. Recently, a role for Nrp1 in T_{reg} cells was proposed to limit tumour growth in the MT/*ret* murine melanoma model[29], although contrary to the findings in that study we did not observe any differences in T_{reg}-cell prevalence in B16 melanoma tumours (Fig. 2h). However, further studies will be required to delineate in which tumours and under what conditions these two disparate functions of Nrp1 in T_{reg} cells—regulation of T_{reg}-cell migration and the maintenance of T_{reg}-cell survival and stability—are used. Our identification of Nrp1–Sema4a as a pivotal pathway required for intratumoral T_{reg}-cell stability, but dispensable for the maintenance of immune homeostasis, suggests that Sema4a–Nrp1 blockade via antibodies or soluble antagonists may be a viable therapeutic strategy to limit tumour-induced tolerance without evoking autoimmunity.

METHODS SUMMARY

Mice. C57/BL6 and dnTGFßRII mice were purchased from the Jackson Laboratories. $Foxp3^{YFP\text{-}iCre}$, $Foxp3^{-}$ and $Foxp3^{DTR\text{-}GFP}$ mice were obtained from A.Y. Rudensky. $Il10^{-/-}$ mice were obtained from T. Geiger. $Nrp1^{f/f}$ mice were obtained from D. Cheresh. $Pten^{f/f} \times Foxp3^{YFP\text{-}iCre}$ mice were obtained from H. Chi. Animal experiments were performed in American Association for the Accreditation of Laboratory Animal Care-accredited, specific-pathogen-free facilities in the St Jude Animal Resource Center. Animal protocols were approved by the St Jude Animal Care and Use Committee.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions G.M.D. designed and performed most of the experiments and wrote the manuscript. S.-R.W. performed critical initial experiments and identified Sema4a and Nrp1 as the ligand–receptor pair. M.E.T. conducted many of the tumour experiments. D.M.G. performed a substantial portion of the colitis experiments. C.G. performed TIRF microscopy. M.L.B. assisted with the *Foxp3*-deficiency rescue experiments. A.E.O. assisted with several experiments. P.V. performed histological analysis. D.F. performed computational analysis of the microarray data. J.B. provided the blocking monoclonal antibodies to Sema4a and Nrp1. C.J.W. conducted and curated the initial microarray analysis. D.A.A.V. conceived the project, directed the research and wrote the manuscript. All authors edited and approved the manuscript.

Author Information The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE41185. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.A.A.V. (vignali.lab@stjude.org).

LETTER

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Interactome map uncovers phosphatidylserine transport by oxysterol-binding proteins

Kenji Maeda[1], Kanchan Anand[1]†, Antonella Chiapparino[1], Arun Kumar[1], Mattia Poletto[1], Marko Kaksonen[1] & Anne-Claude Gavin[1]

The internal organization of eukaryotic cells into functionally specialized, membrane-delimited organelles of unique composition implies a need for active, regulated lipid transport. Phosphatidylserine (PS), for example, is synthesized in the endoplasmic reticulum and then preferentially associates—through mechanisms not fully elucidated—with the inner leaflet of the plasma membrane[1–3]. Lipids can travel via transport vesicles. Alternatively, several protein families known as lipid-transfer proteins (LTPs) can extract a variety of specific lipids from biological membranes and transport them, within a hydrophobic pocket, through aqueous phases[4–7]. Here we report the development of an integrated approach that combines protein fractionation and lipidomics to characterize the LTP–lipid complexes formed *in vivo*. We applied the procedure to 13 LTPs in the yeast *Saccharomyces cerevisiae*: the six Sec14 homology (Sfh) proteins and the seven oxysterol-binding homology (Osh) proteins. We found that Osh6 and Osh7 have an unexpected specificity for PS. *In vivo*, they participate in PS homeostasis and the transport of this lipid to the plasma membrane. The structure of Osh6 bound to PS reveals unique features that are conserved among other metazoan oxysterol-binding proteins (OSBPs) and are required for PS recognition. Our findings represent the first direct evidence, to our knowledge, for the non-vesicular transfer of PS from its site of biosynthesis (the endoplasmic reticulum) to its site of biological activity (the plasma membrane). We describe a new subfamily of OSBPs, including human ORP5 and ORP10, that transfer PS and propose new mechanisms of action for a protein family that is involved in several human pathologies such as cancer, dyslipidaemia and metabolic syndrome.

To determine LTP specificity, we purified LTP–lipid complexes from extracts of yeast strains expressing physiological amounts of LTPs fused to the tandem-affinity purification (TAP) tag (Supplementary Fig. 1a)[8,9]. This approach combines high-affinity purification using an immunoglobin G (IgG) resin, elution with a site-specific protease, and separation by size-exclusion chromatography (SEC). Proteins and lipids in the SEC fractions were then analysed by denaturing gel electrophoresis (SDS–PAGE) and high performance thin layer chromatography (HPTLC) or mass spectrometry, respectively (Supplementary Figs 2–4). The SEC elution profiles discriminate any background binding to IgG and only lipids co-eluting with LTPs were considered to represent specific ligands. Importantly, the procedure allows LTPs to be maintained in conditions that closely approximate normal physiology and preserves their ability to form multiprotein complexes. For example, Sec14 was found to co-elute with a known interaction partner Ptc7 (systematic name Yhr076w), a 38-kDa type 2C protein phosphatase (Supplementary Fig. 3). The analysis confirmed many other known or predicted interactions, such as the ones between Kes1 (also known as Osh4, systematic name Ypl145c) and sterol[10] and between Sec14 (systematic name Ymr079w) or Sfh1 (systematic name Ykl091c; 64% primary sequence identity with Sec14) and phosphatidylinositol (PI)/phosphatidylcholine (PC)[11](Supplementary Figs 1b, 2 and 3).

Among proteins with an OSBP domain, Osh6 (systematic name Ykr003w) and Osh7 (systematic name Yhr001w)—two paralogues that have poor sterol-transfer activity *in vitro*[12]–formed stoichiometric and specific complexes with PS but not with sterols (Supplementary Figs 1c, 2 and 4). For Osh6, the amount of PS in the pull-downs was ~100-fold higher than in the controls Kes1 and Sec14, no other lipids co-eluted at significant levels and the protein only associated with PS species with long aliphatic chains (C34) (Supplementary Figs 1d and 4). Osh6 and Osh7 seem to participate in lipid metabolic pathways *in vivo* that are distinct from those of other OSBPs. Kes1 fused to green fluorescent protein (GFP) localizes to the cytosol[13], whereas Osh6–GFP resides in structures at the cell periphery that overlap with cortical endoplasmic reticulum (ER) and represent ER–plasma membrane (PM) contact sites[12](Supplementary Fig. 5a). Upon perturbations of either ergosterol or PI phosphate metabolism, Kes1–GFP translocated to juxtanuclear patches[13], whereas only mutations targeting PS metabolism specifically affected Osh6–GFP localization at the cell periphery (Supplementary Fig. 5b). These data indicate a new role for the OSBP family in the transport of PS, an important signalling lipid. This forms the basis for the more detailed mechanistic and structural studies discussed below.

To confirm the interaction between Osh6 and PS, we produced Osh6 in *Escherichia coli*. The recombinant Osh6 co-purified with phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), two abundant lipids in bacteria, and with PS (Supplementary Fig. 6a). These complexes were resistant to exposure to a non-ionic detergent, indicating that the ligands are poorly accessible. We measured Osh6 lipid-binding specificity *in vitro* using liposomes containing PS, PG, PE, PI, cardiolipin (CA), ergosterol or mixtures of lipids originating from yeast membranes (in which PG exists in trace amounts[14]). We found that only PS bound efficiently to recombinant Osh6 and that PS could replace bacterially produced and co-purifying PG and PE (Fig. 1a, b). As a control in the same assay, recombinant Kes1 bound ergosterol, but not PS. This shows that *in vitro*, Osh6 specifically binds PS and exchanges lipids with membrane bilayers.

We then determined whether Osh6/Osh7 can transfer PS between membranes *in vitro*. For this purpose, we used either recombinant Osh6 (Fig. 1c and Supplementary Fig. 6b) or native Osh6–TAP and Osh7–TAP (Fig. 1d) purified from yeast. We designed two different lipid-transfer assays. First, we monitored the transfer of lipids from 'heavy' donor liposomes (that contained different putative lipid cargoes) to 'light' acceptor liposomes (Fig. 1c, d). The donor liposomes were filled with sucrose so that they could readily be separated by centrifugation[12]. In the second assay, we measured the amount of PS transferred from donor to immobilized and fluorescently labelled (green) acceptor liposomes. We visualized the presence of PS in the acceptor liposome with a specific probe, the fluorescently labelled annexin V (red, Cy3)(Supplementary Fig. 6b). Overall, the data demonstrate that *in vitro* both Osh6 and Osh7, but neither Kes1 nor Sec14, specifically transfer PS between donor and acceptor membranes.

Osh6 and Osh7 localize at membrane contact sites that bridge PS synthesis (the ER) with areas of PS accumulation and biological

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[1] European Molecular Biology Laboratory, EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany. †Present address: Center of Advanced European Studies and Research (Caesar), Ludwig-Erhard-Allee 2, 53175 Bonn, Germany.

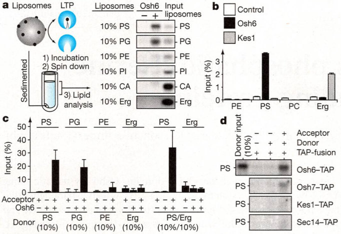


Figure 1 | Recombinant Osh6/Osh7 bind and specifically transfer PS *in vitro.* **a**, Osh6 specifically associates with phosphatidylserine (PS) and phosphatidylglycerol (PG). Osh6-associated lipids were determined by HPTLC. Liposomes (2% of the input) were loaded as references. **b**, Osh6 extracts PS from liposomes containing yeast total lipid extract. Kes1 associates with ergosterol (Erg). Control: without lipid-transfer proteins (LTPs). **c**, Recombinant Osh6 specifically transfers PS from heavy donor liposomes to light acceptor liposomes. **d**, Transfer of PS from the donor to acceptor liposomes by Osh6–TAP and Osh7–TAP purified from yeast. CA, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol. Error bars represent standard deviation, $n \geq 3$.

activity (the PM)[12](Supplementary Figs 5a and 7a). We first examined whether Osh6 and Osh7 contribute *in vivo* to the subcellular compartmentalization of PS by measuring the content of the lipid in different subcellular membranes (Fig. 2a and Supplementary Fig. 7a). In wild-type cells, PS is more concentrated in the PM- than in the ER-enriched fractions. By contrast, in the absence of Osh6/Osh7, PS accumulation at the PM was reduced (~30%), whereas the distribution of other glycerophospholipids was largely unaffected. We also performed additional tests using an *in vivo* probe for cellular PS: the lactadherin C2 domain fused to GFP (Lact C2–GFP)[1]. Consistent with the biochemical data, PS predominantly localized at the PM in the unperturbed, *osh6Δ*, *osh7Δ* and *kes1Δ* (control) strains[2], where it laterally partitioned into domains (Fig. 2b and Supplementary Fig. 7a). Deletion of both Osh6 and Osh7 induced PS delocalization from the PM and the Lact C2–GFP probe adhered to intracellular membrane structures representing the ER and vacuoles (Fig. 2b and Supplementary Fig. 7a, b). PS mislocalization was rescued by the reintroduction of wild-type Osh6, but not the mutants Osh6 Lys126Ala or Leu69Asp that are deficient in PS binding (see below; Supplementary Fig. 7c) or of an engineered Osh6 that localizes at vacuolar membranes (see below; Supplementary Fig. 7d). Collectively, these results indicate that *in vivo*, active Osh6/Osh7 at the ER–PM contact sites contribute to the delivery and accumulation of PS at the PM.

PS is also transported from the ER to sites of PS decarboxylation, where PS serves as a precursor for the biosynthesis of PE and PC (Fig. 2c). If Osh6 and Osh7 contribute to PS decarboxylation, then their deletions should lead to a drop in the cellular levels of PE/PC. However, in *osh6Δosh7Δ* cells, the total amounts of PE/PC were largely unaffected (Fig. 2a). We observed instead a significant decrease in PS levels that could be reversed by the deletion of the main decarboxylase in yeast, the mitochondrial Psd1 or the addition of choline (a precursor for the Kennedy pathway, that is, an alternative, PS-independent route of PC synthesis). These results disprove a major role for Osh6/Osh7 in the delivery of PS to the main sites of decarboxylation in yeast[10]. This is also consistent with the notion that Osh6/Osh7 contribute to the accumulation of PS at the PM so that it avoids decarboxylation in the mitochondria. Our data support the view that there are distinct routes for regulated PS delivery to its different sites of biological activity (Fig. 2c). Cellular processes implying intracellular PS transport, that is, PS decarboxylation into PE (mitochondria) or the vesicular trafficking-, cell cycle-dependent polarization of PS at the buds[3,15], were largely unaffected in *osh6Δosh7Δ* strains (data not shown).

We then investigated whether Osh6/Osh7 directly transport newly synthesized PS between cellular membranes *in vivo*. We first depleted endogenous PS by deleting the PS synthase (Cho1, systematic name Yer026c). Lyso-PS (derivatives of phosphatidylserine in which one acyl chain have been removed by hydrolysis) subsequently added to the media was incorporated into the cells and was rapidly converted to PS in the ER (wild-type cellular levels of PS were restored within 10 min; Supplementary Fig. 8a)[16,17]. We were then able to measure the kinetics of Osh6/Osh7-mediated transfer of newly synthesized PS using the Lact C2–GFP probe[1]. In the absence of Osh6/Osh7, or in the presence of a mutated Osh6 deficient in PS binding (Leu69Asp; see below), newly formed PS remained trapped in the ER (Fig. 2d and Supplementary Fig. 8b, c). By contrast, functional Osh6/Osh7 triggered a rapid (within 2 min) translocation of PS from the ER to the PM. The rate at which newly synthesized PS equilibrates in the PM was slower in the Osh6/Osh7 double mutant than in wild-type cells (Supplementary Fig. 8b). Osh6/Osh7-mediated PS transfer took place at 4 °C, further indicating that PS transport is largely independent of vesicular trafficking. In *osh6Δosh7Δ* strains at 4 °C, a small fraction of the Lact C2–GFP probe remained bound to the PM, indicating that additional, Osh6/Osh7-independent, non-vesicular mechanisms of PS transport may exist. Finally, we also examined whether the translocation of Osh6 to other cellular membranes is sufficient to redirect PS to this new location (Fig. 2e, f). In an Osh7-knockout strain, we co-expressed Osh6 fused to human FKBP (FK506-binding protein) and Vph1 (a trans-membrane, vacuolar protein) fused to human FRB (FKBP-rapamycin-binding domain). Rapamycin induced the relocalization of Osh6–FKBP to vacuoles, particularly to regions adjacent to the ER (Fig. 2e). Upon Osh6 translocation, newly synthesized PS also became quickly (within 5 min) redirected to this organelle (Fig. 2f), indicating that Osh6/Osh7 can directly transport PS between cellular membranes *in vivo*. Together, these results provide the first direct evidence for non-vesicular transfer of PS from its site of biosynthesis (the ER) to its site of biological activity (the PM). We propose that members of the Osh protein family are integral parts of the cellular machinery regulating PS homeostasis and signalling and are responsible for its accumulation at the PM.

To gain insights into the mechanisms for specific PS recognition, we determined the structure of the Osh6–PS complex by X-ray crystallography at 1.95 Å resolution (Supplementary Fig. 9a and Supplementary Table 1). Similar to Kes1[18,19] (20% sequence identity to Osh6), the Osh6 structure consists of an incomplete β-barrel that forms the ligand-binding tunnel (residues 74–327), an amino-terminal 'lid' (residues 35–73) covering the tunnel entrance and a carboxy-terminal mainly α-helical region that does not contact the ligand (residue 328–434) (Supplementary Fig. 9a). The structure also revealed a clear electron density map in the ligand-binding pocket of Osh6 in which we could model PS with a 17 carbon-long saturated acyl chain (*sn*-1 position) and an 18 carbon-long unsaturated acyl chain (oleic acid; *sn*-2 position). The Osh6 tunnel, which can accommodate the long aliphatic chain of PS, is predominantly hydrophobic and is about 8–10 Å deeper than the one reported for Kes1 (Supplementary Fig. 9b)[18,19]. The head group and the *sn*-2 acyl group of PS are oriented towards the tunnel entrance and the *sn*-1 acyl group towards the bottom of the tunnel (Supplementary Fig. 9a). The entire PS molecule is involved in extensive interactions with 36 residues from Osh6 (Fig. 3a). In particular, the α1-β1 loop in the lid and the region near the tunnel entrance are crucial for PS recognition (see below). This implies a series of hydrophobic residues in the α1-β1 loop (Leu 64, Ile 67, Leu 69 and Ile 73) and at the tunnel entrance (Val 124) that form

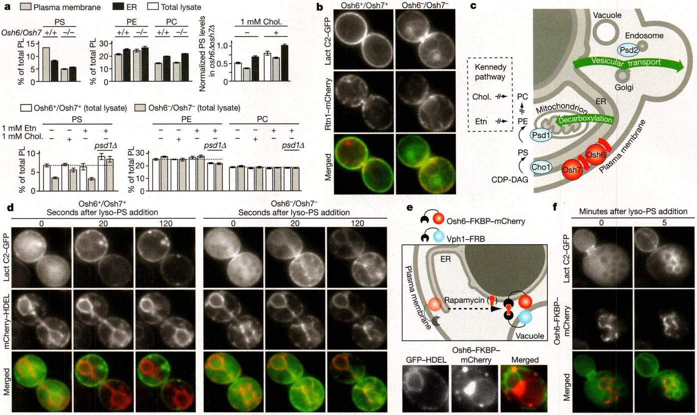
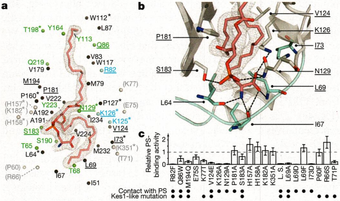


Figure 2 | ***In vivo*, Osh6/Osh7 transport PS from the ER to the PM.** **a**, Lipidomics analyses (glycerophospholipid, PL) of PM or ER fractions (top), and of total cell extracts (bottom). **b**, Osh6/Osh7 are required for the accumulation of PS to the PM. Rtn1–mCherry marks the ER. **c**, Schematic view of the role of Osh6/Osh7 in PS trafficking. CDP-DAG, cytidine diphosphate-diacylglycerol. **d**, Kinetics of Osh6-mediated PS transfer *in vivo*. Lact C2–GFP re-localization after the addition of 18:1 lyso-phosphatidylserine (Lyso-PS) in PS-depleted cells (*cho1Δ*). mCherry–HDEL marks the ER. **e**, Rapamycin induces Osh6–FKBP–mCherry to dimerize with Vph1–FRB and to translocate to vacuolar membranes adjacent to the ER. GFP–HDEL marks the ER. **f**, The vacuolar translocation of Osh6 is followed by an accumulation of newly synthesized PS (from lyso-PS) in vacuoles. Error bars represent standard deviation, $n \geq 3$. Chol., choline; Etn, ethanolamine.

extensive non-polar contacts with the *sn*-2 acyl group of PS (Fig. 3b). Several residues in the α1-β1 loop (Leu 64, Ile 67 and Leu 69) and at the tunnel entrance (Lys 126, Asn 129 and Ser 183) are also engaged in polar interactions with the PS head group, especially with the carboxylate anion. This could explain the specificity of Osh6 for PS versus the related PE and PC, which are products of PS decarboxylation (Supplementary Fig. 10).

Only nine of the PS-interacting residues in Osh6 are conserved in Kes1. Not surprisingly, Osh6 in which these conserved interacting residues are mutated—including Lys126Ala, Asn129Ala (polar contacts with the PS carboxylate anion) or Ile73Asp (hydrophobic contact with PS)—binds very poorly to PS *in vitro* (Fig. 3c), indicating that they have conserved roles in ligand binding. By contrast, mutations of conserved, polar amino acids located near the PS head group, but not interacting with it (His 157, His 158, Lys 351 and Lys 182), had no effect. Importantly, most Osh6 residues that bind to PS are not conserved in Kes1 and probably give rise to Osh6 specificity. In particular, the α1-β1 loop in the lid region, which is poorly conserved in Kes1 (where it partially folds as a short 3/10 helix[18,19]), makes extensive polar and non-polar contacts to PS (Fig. 3b). Osh6 in which Leu 69 is substituted for a charged residue (Asp) or for the corresponding Kes1 residue (Ala) interacted poorly with PS, whereas substitution for another hydrophobic residue (Phe) had no effect (Fig. 3c). Further supporting the notion that the α1-β1 loop has a key role in specific PS binding, additional mutations of Thr 71 (to Pro) or the substitution of the entire α1-β1 loop with the corresponding loop of Kes1 completely abrogated the association with PS. In total, we replaced six Osh6-specific, PS-contacting residues (Leu 69, Arg 82, Gln 86, Val 124, Ser 183 and Met 194) with the corresponding ones in Kes1 and for five of these, this led to significant decreases in PS binding (Fig. 3c). The crystal structure of Osh6 demonstrates that binding to PS involves a series of amino acids that are not conserved in Kes1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W112*
L87
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Q86
V83
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R82
V179
M194
M79
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P181
P160*
V222
(H157*)
Y223
N129*
P127*
(E75)
(K182*)
A192
K126*
K125*
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A191
I234
V124
S183
V224
I73*
S190
(K351*)
T65
M232
(T71)
L64*
L69
(P60)
T68
(R66)
I67
I51
b
V124
K126
P181
I73
S183
N129
L69
L64
I67
c
Relative PS-binding activity
2
1
0
R82F
Q86W
M194Q
E75S
K77E
V124E
K126A
N129A
P181A
S183A
H157A
H158A
K182A
K351A
L.S.
L69A
L69D
L69F
I73D
P60F
R66S
T71P
Contact with PS
Kes1-like mutation

Figure 3 | **Structure of the Osh6–PS complex.** **a**, An overview of Osh6–PS interactions. Cα atoms (spheres) of polar (green), positively charged (blue) and hydrophobic (black) residues that form contacts to PS (sticks). Underlined, mutated residues. Grey, mutated residues not forming contact. Light grey isomesh map: PS $2F_o - F_c$ electron density map at the 1.0σ level. Stars, residues conserved in Kes1. **b**, Close-up of region forming key contacts with PS (sticks). Blue, α1-β1 loop. Dashed lines, hydrogen bonds <3.5 Å. **c**, *In vitro* PS-binding activities of Osh6 mutants. Kes1-like mutants introduce corresponding Kes1 amino acids. The 'loop-swap' (L.S.) mutant: Osh6 α1-β1 loop was swapped for the corresponding segment in Kes1. Error bars represent standard deviation, $n \geq 3$.

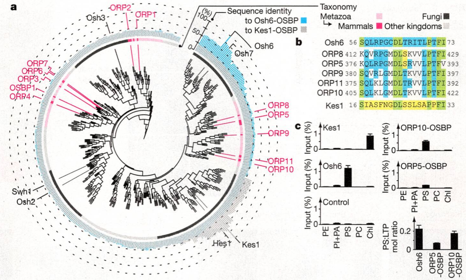

Figure 4 | Osh6/Osh7 are the first representatives of a PS-binding OSBP subfamily conserved in humans. a, A phylogenetic analysis of all OSBP domains. Black, *S. cerevisiae* OSBPs; magenta, human OSBPs. Bar graphs, sequence identities derived from pair-wise alignment with Osh6-OSBP (blue) and Kes1-OSBP (grey). **b,** Sequence features of the Osh6 α1-β1 loop are conserved in the five clustered human OSBP domains. Blue and yellow: Osh6- and Kes1-specific residues, respectively. Green, residues common to both. **c,** The OSBP domains of ORP5 and ORP10 bind PS *in vitro*. OSBP-bound fractions of individual lipids are quantified. Bottom: the PS:OSBP molar ratios for recombinant Osh6, ORP5-OSBP and ORP10-OSBP. Chl, cholesterol. Error bars represent standard deviation, $n \geq 3$.

and may represent the signature of a new subfamily of OSBPs with specificity for PS.

Our results show that in yeast, Osh6 and Osh7 define a unique OSBP subfamily with structural features adapted for PS recognition. To determine whether this subfamily is conserved in other kingdoms, we performed a phylogenetic analysis with all OSBP domains entered in the Pfam database[20] (see Methods). The seven OSBP domains of Osh proteins in *S. cerevisiae* distribute broadly across the entire tree (Fig. 4a). Those of Osh6/Osh7 and Kes1 belong to two clades, consistent with the view that they define distinct subfamilies. Importantly, the Osh6/Osh7 clade also contains OSBP domains in metazoans, including five human proteins, ORP5, ORP8, ORP9, ORP10 and ORP11, suggesting a broad conservation across eukaryotes. In particular, we observed a high degree of conservation of the sequence signature that is required for Osh6 to bind specifically to PS (see above)(Fig. 4b). Within the α1-β1 loop, we could define sequence motifs characteristic of the Osh6/Osh7 and Kes1 subfamilies (Supplementary Fig. 11). We selected two human OSBP domains that clustered within the Osh6/Osh7 subfamily, one from ORP5 and one from ORP10, and measured their binding specificity *in vitro*, using liposomes derived from brain total lipid extract. We observed that the recombinant OSBP domains from ORP5 and ORP10 efficiently and specifically extracted PS (Fig. 4c). This supports the notion that Osh6 and Osh7 are the first representatives of a broader subfamily of OSBPs that transport PS and which are also conserved in humans.

Other members of the OSBP family are known to bind sterols and PI lipids[19]. In humans, many are involved in diseases[21,22] and in yeast they share a common, essential function, as only one of the seven proteins is required for yeast survival[23].We demonstrate that PS transport represents a new function for some members of this protein family, but clearly, binding to a specific lipid is not a common and essential task. *De facto*, the name 'oxysterol-binding protein' is a misnomer that should be revised and we propose that 'lipid sensing and sorting' (Lss) protein could be more appropriate. Several models could account for the Osh6/Osh7-mediated PS transport against an apparent concentration gradient. A likely scenario relies on the fact that PS associates with specific PM microdomains[17,24]. This may contribute to its sequestration from the pool of 'free' PS. Consistent with this hypothesis, we observed that Osh6 and PS (Lact C2–GFP) partition in spatially discrete domains with distinct dynamic properties (Supplementary Fig. 7a). In addition, in the ER, the spatial confinement of PS-synthesizing enzymes, for example, Cho1, at PM contact sites[25] may produce high, local PS concentrations that also contribute to the transfer of PS from ER to the PM. An important outcome of our study is the ease by which *in vivo*-assembled LTP–lipid complexes can be retrieved from cells. This work clearly demonstrates the feasibility of broader efforts that would include all LTPs in higher eukaryotes[26–30].

METHODS SUMMARY

Purification of LTP–TAP fusions and lipid analysis by HPTLC. The TAP fusions were affinity purified on IgG agarose resins and eluted with 20 µg tobacco etch virus protease (TEV) at 4 °C. The eluates (100 µl) were loaded on a gel filtration column and 150 µl fractions were collected. Lipids were extracted from samples by sequential addition of solvents. The lipid fraction was sprayed on HPTLC silica gel 60 plates that were developed with solvent systems for neutral lipids and phospholipids. The plates were dripped in 10% (w/v) CuSO4 in 8% (v/v) aqueous phosphoric acid, charred at 145 °C for 4.5 min, and scanned for fluorescence detection at 488 nm extinction and 530 nm emission wavelengths.

Crystallization of Osh6–PS complex. The purified Osh6 was treated with tenfold molar excess of PS. The Osh6–PS complexes were purified with Ni-NTA resins followed by gel filtration, and crystallized using vapour diffusion techniques. Diffraction data were collected at beamline ID23-1 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The structure was solved by molecular replacement.

The protocols for the production of recombinant proteins, the *in vitro* binding and transfer assays, the cell fractionation, lipidomics, the visualization of intracellular PS and the phylogenetic analysis of OSBP domains are described in the Methods section.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions K.M. and A.-C.G. designed the research; K.M., A.C. and A.K. conducted the experiments and performed the analysis; K.A. and K.M. performed X-ray crystallography; M.P. supported the development of the biochemical protocols; M.K. provided technical expertise with instrumentation; and K.M. and A.-C.G. discussed results and wrote the manuscript with support from all the authors.

Author Information The atomic coordinates and structure factors have been deposited in the Protein Data Bank at entry 4B2Z. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.-C.G. (e-mail: gavin@embl.de).

CORRECTIONS & AMENDMENTS

CORRIGENDUM

doi:10.1038/nature12498

Corrigendum: A CRISPR/Cas system mediates bacterial innate immune evasion and virulence

Timothy R. Sampson, Sunil D. Saroj, Anna C. Llewellyn,
Yih-Ling Tzeng & David S. Weiss

Nature **497,** 254–257 (2013); doi:10.1038/nature12048

In this Letter, we described two small RNAs (scaRNA and tracrRNA) within the *Francisella novicida* CRISPR/Cas locus that are necessary for repression of an endogenous transcript. Concurrent to our studies, E. Charpentier's group performed RNA sequencing analyses of multiple type II CRISPR loci to identify tracrRNA in *F. novicida* and other species[1], based in part on the co-processing of tracrRNA:crRNA by RNase III (ref. 2). These observations indicate that the regulatory RNA we annotated as scaRNA is the tracrRNA, and that the RNA we annotated as tracrRNA is the scaRNA. Furthermore, RNAseq data show that the transcriptional direction of the crRNA array is the opposite of what was predicted. Finally, our predictions of tracrRNA and scaRNA within Supplementary Table 2 are incorrect based on this transcriptional analysis; the scaRNAs predicted in *Neisseria meningitidis* 92045, *Listeria monocytogenes* SLCC2482 and *Streptococcus pyogenes* M1 GAS are in fact within the crRNA array transcript, as is the predicted tracrRNA in *Campylobacter jejuni* NCTC11168, whereas the predicted *C. jejuni* scaRNA is actually the tracrRNA. We include a corrected annotated Fig. 1a below, and apologize for any confusion about our incorrect nomenclature. We are grateful to E. Charpentier and her group for alerting us to the errors.

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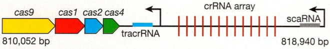

Figure 1 | This is the corrected Fig. 1a of the original Letter.

RETRACTION

doi:10.1038/nature12497

Retraction: Bird-like fossil footprints from the Late Triassic

Ricardo N. Melchor, Silvina de Valais & Jorge F. Genise

Nature **417,** 936–938 (2002); doi:10.1038/nature00818

In this Letter, we considered the bird-like footprints from the former Santo Domingo Formation of northwest Argentina to be of Late Triassic age. Recent radiometric dating[1] of the sedimentary sequence containing these bird-like footprints (renamed as the Laguna Brava Formation) indicated a Late Eocene age. Further geological studies[2] suggest that the region suffered a complex deformation during the Andean orogeny, including block rotation. In consequence, our previous inferences about the possible implications of this finding for the fossil record of Aves are no longer supported. This Retraction has not been signed by J.F.G. Correspondence should be addressed to R.N.M. (rmelchor@exactas.unlpam.edu.ar).

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CORRIGENDUM

doi:10.1038/nature12527

Corrigendum: X-ray analysis on the nanogram to microgram scale using porous complexes

Yasuhide Inokuma, Shota Yoshioka, Junko Ariyoshi,
Tatsuhiko Arai, Yuki Hitora, Kentaro Takada,
Shigeki Matsunaga, Kari Rissanen & Makoto Fujita

Nature **495,** 461–466 (2013); doi:10.1038/nature11990

Previously unnoticed ambiguities in the crystallographic data in our Article (specifically, non-negligible disorder of the miyakosyne A molecule), along with further work by three of the authors (Y.H., K.T. and S.M.) have revealed that the stereochemistry we assigned at C14 of miyakosyne A is incorrect. Our new investigations confirm that we can indeed determine the molecular skeleton of miyakosyne A. However, we can only tentatively and not unambiguously identify all of the stereochemistry of miyakosyne A based on the data included in the original paper. A future publication will confirm the stereochemistry at the C14 moiety of miyakosyne A. The other conclusions of our paper are not affected by this correction.

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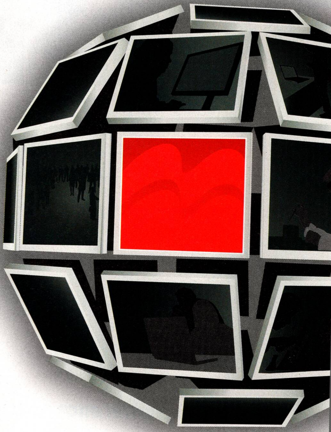

TECHNOLOGY FEATURE

THE GENOME JIGSAW

Advances in high-throughput sequencing are accelerating genomics research, but crucial gaps in data remain.

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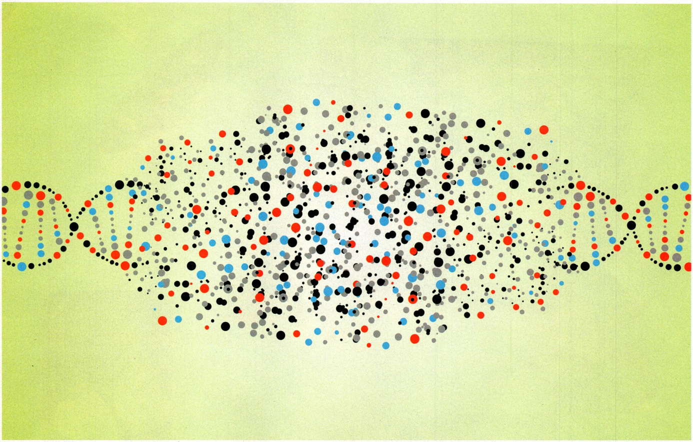

BY VIVIEN MARX

To understand why high-throughput gene-sequencing technology often produces frustrating results, says Titus Brown, imagine that 1,000 copies of Charles Dickens' novel *A Tale of Two Cities* have been shredded in a woodchipper. "Your job is to put them back together into a single book," he says.

That task is relatively easy if the volumes are identical and the shreds are large, says Brown, a microbiologist and bioinformatician at Michigan State University in East Lansing. It is harder with smaller shreds, he says, "because if the sentence fragments are too small, then you can't uniquely place them in the book". There are too many ways they might fit together. "And it's harder still if the original pile of books includes multiple editions," he says.

Researchers in genetic sequencing today face a similar task. An organism's DNA — made up of four basic building blocks, or bases, denoted by the letters A, T, C and G — is chopped into short snippets, sequenced to determine the order of its bases and reassembled into what researchers hope is a good approximation of the organism's actual genome.

Today's high-throughput sequencing technology is remarkably powerful and has led to an explosion of sequencing projects in laboratories around the world, says Jay Shendure, a molecular biologist who develops sequencing methods at the University of Washington School of Medicine in Seattle. Thousands of patient tumours and more than 10,000 vertebrate species have been or are being sequenced. High-throughput sequencing is now an essential tool for basic and clinical research, with applications ranging from detection of microbial 'bio-threats' to finding better biofuels[1].

But some types of genomic DNA cannot be sequenced by high-throughput methods, leaving many frustrating gaps in data (see 'What makes a tough genome?'). For example, a genome might contain long stretches in which the sequence simply repeats — as if Dickens had filled whole pages with a word or sentence

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written over and over — making that passage hard, if not impossible, to reconstruct by the usual technologies. And the widespread adoption of next-generation sequencing has meant that the quality of genome assemblies has declined significantly over the past six years, says Evan Eichler, a molecular biologist also at the University of Washington. Although "we can generate much, much more sequence, the short sequence-read data translate into more gaps, missing data and more incomplete references," he says.

Incomplete genomes make it harder for researchers to identify and interpret sequence variations. "Instead," Eichler says, "we focus only on the accessible portions, creating a biased view," which in turn hinders efforts to study the genetic basis of disease or how species have evolved. For example, the human-genome sequence, used as a reference by scientists around the world, has more than 350 gaps, says Deanna Church, a genomicist at the US National Center for Biotechnology Information. An updated reference genome is filling in much of the missing data, but "even with the release of the new assembly, there will still be gaps and regions that aren't well represented," she says. "It is definitely a work in progress."

More than 900 human genes are in regions where there is much repetition. About half of these genes are in areas so poorly understood that they are often excluded from biomedical study, says Eichler. Certain regions of chromosomes, notably those near centromeres (where the two halves of a chromosome connect) and telomeres (the ends of chromosomes) are especially incomplete in the reference genome.

UNIV. WASHINGTON

Jay Shendure is working to develop the next generation of sequencing methods.

This lack of information can have medical consequences. For example, researchers have known for more than a decade that medullary cystic kidney disease — a rare disorder that occurs in mid-life — can be caused by mutations in a gene hidden somewhere along a 2-million-base-pair stretch of chromosome 1. Early detection of the mutation is the first step towards preventative therapies, but would require a DNA test. The gene, however, lies within a region rich in sequence repeats as well as in the bases guanine (G) and cytosine (C). Such 'GC-rich' regions, like repetitions, are difficult to sequence.

Only by reverting to Sanger sequencing — a classic but more laborious approach — and combining it with special assembly methods were researchers able to decipher the DNA region involved in the disease. The results, which were published in February[2], mean that a test to screen younger members of families affected by the disorder is now a possibility.

Sanger sequencing is a painstaking process in which each type of DNA base is labelled with a different compound. The labelled DNA is then separated and the sequence is read. For the Human Genome Project, researchers combined Sanger sequencing with techniques to establish markers that locate where the sequences fit. The approach, which has been in use for decades, delivers a read accuracy and contiguity of sequence that are unmatched by current technology, Shendure says. "I couldn't do anything remotely approaching the quality of what resulted from the project." But the art of Sanger sequencing and its associated methods cannot be scaled up for the high-throughput sequencing projects done today. "We need to think about how to 'next-generation-ify' all of this," he says.

Research to do just that is well under way, with a variety of methodologies that address problems such as repetitive sequences and GC-rich regions, as well as the knotty task of assembling complete genomes for organisms that have four or even eight copies of each chromosome, for example, as opposed to humans' two.

Some of the technologies on the horizon promise to deliver longer reads and, possibly, fewer headaches for researchers trying to assemble them. But until those instruments are on bench tops, scientists are combining new and old approaches to refine sequencing.

RICH IS POOR

Some of the newer approaches aim to tackle GC-rich regions. For high-throughput sequencing, DNA is often first chopped into short fragments, which are then amplified by polymerase chain reaction (PCR). But the enzyme used in PCR "has trouble getting through" GC-rich regions, says Shendure. As a result, GC-rich stretches can end up poorly represented in the DNA sample delivered to the sequencer, thus skewing the data. Some sequencing technologies, such as those made by Illumina, based in San Diego, California, use amplification before and during the sequencing process, causing further bias against GC regions.

A number of sample-preparation approaches reduce this GC bias. The amplification step is cut out completely in platforms made by Pacific Biosciences, based in Menlo Park, California, and in a method being developed at Oxford Nanopore Technologies in Oxford, UK. And although DNA read lengths differ among platforms, the most widely used bench top sequencers — which are made by Illumina — generate short reads, of around 150 base pairs.

"The killer with short reads is that they're very sensitive to repeated content," says Brown. If the read length is shorter than a repeat — or, to draw on the book analogy, if the shreds of the novel are only a fraction as long as a repeated paragraph — it is hard or even impossible to uniquely place. "That's where things like long reads or other technologies can be helpful," says Shendure. Long DNA fragments can bridge repetitive regions and thus help to map them. As another way to ease assembly, researchers in Shendure's group and elsewhere are exploring different methods to tag and group DNA fragments before sequencing. "There are more on the horizon," says Shendure, but he prefers to divulge the details in research publications.

What makes a tough genome?

Certain features of DNA are challenging for high-throughput sequencing.

- Long sequences of repeated bases
- Missing bases in the original sequence
- Degraded or damaged DNA
- Regions rich in guanine and cytosine

The terms 'short' and 'long' are in a state of flux in this fast-moving industry. The first generation of Illumina machines generated reads of around 25 base pairs in length; the latest ones have upped that to around 150 base pairs (see 'Extended sequence'). But it is still hard to assemble a complete genome from reads of this length.

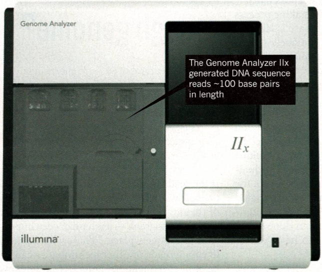
Geoff Smith, who directs technology development at Illumina in Cambridge, UK, acknowledges the drawbacks of short-read technology for sequencing repetitive regions and various types of genomic rearrangements. He says that the company aims to address issues that crop up as researchers compare genomes they sequence to reference genomes, or sequence organisms from scratch without references.

Illumina has launched a service to allow longer reads with its current short-read technology. Last year the firm bought Moleculo, a company based in San Francisco, California, which has developed a process to create long reads by stitching together short ones through a proprietary sample-preparation and computational process. In July Illumina began offering Moleculo's process as a service for customers.

The Moleculo process first creates DNA fragments about 10,000 bases (10 kilobases) in length. The fragments are sheared and amplified, then grouped and tagged with a unique barcode that helps to identify which larger fragment they originated from and aids in assembly.

EXTENDED SEQUENCE

Illumina's first high-throughput sequencers produced very short reads.



The Genome Analyzer IIx generated DNA sequence reads ~100 base pairs in length

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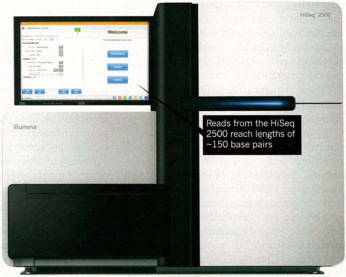


Reads from the HiSeq 2500 reach lengths of ~150 base pairs

2013

ILLUMINA

At present, sample preparation for the Moleculo process takes around two days. Smith says that he and his team are refining the process and that by the end of the year Illumina will launch Moleculo as a stand-alone sample-preparation kit. He says that company scientists are now evaluating the kit's performance by sequencing a well-known genome, but he prefers not to say which one.

"We suspect you will be able to uncover a lot more of the genome with 10-kilobase reads versus the [150-base-pair] read length that we currently have," says Smith. He adds that the company plans to increase the fragment length to 20 kilobases. He and his team hope to "develop better molecular-biology tools to allow us to reach into these difficult-to-sequence parts of the genome but also use those tools on well-characterized genomes," he says. The team is also tuning the Illumina software to better distinguish between false and correct reads.

The company's initiative comes at a time of intense commercial and academic activity around long-read sequencing technology and new assembly methods. Finished genomes have taken a back seat, leaving many highly fragmented assemblies that need completing, says Jonas Korlach, chief scientific officer of sequencing manufacturer Pacific Biosciences in Menlo Park, California, whose sequencer generates read lengths of around 5 kilobases.

Korlach agrees that long reads will help to sequence repetitive regions, such as those that characterize many plant genomes, for example. They will also help with the challenge of distinguishing between copies of chromosomes, important in identifying the tiny variants that can affect biological function. Humans are diploid, meaning they have two copies of each chromosome, but "many organisms, especially plants, have even more copies, which makes resolving all the different chromosomes so much harder", Korlach says.

TOUGH NUTS

Plant sequencing, in particular, will benefit from improvements[3]. The spruce genome is a "real nightmare", says Stefan Jansson, a plant biologist at the Umeå Plant Science Centre in Sweden. Jansson led a study that generated a draft assembly of the Norway spruce genome (*Picea abies*)[4]. In addition to being the largest genome yet sequenced, it also contains many repeats, and the differences between its chromosomes are larger than in the human genome. "Sequencing diploid spruce is like mixing human and chimpanzee DNA and then trying to assemble them simultaneously," Jansson says.

Many plants have more than two copies of chromosomes. Bread wheat (*Triticum aestivum*), for example, is hexaploid, and sequencing and assembling the six sets of chromosomes to completion has proven extremely difficult. And although some strawberry species are diploid, the commercial strawberry (*Fragaria* × *ananassa*) is octoploid: it has eight sets of seven chromosomes, four sets from each parent, says Thomas Davis, a plant biologist at the University of New Hampshire in Durham. "Good thing Mendel didn't use octoploid strawberries to try to understand heredity," he says.

Davis and his colleagues have published a draft genome of the diploid woodland strawberry (*Fragaria vesca*), and now want to apply their experience to the octoploid strawberry[5]. Assembling this tough-nut genome will require high-quality reads longer than 500 base pairs, Davis says. He believes he can succeed, although he does not want to share his methodology just yet. "If anyone cracks that nut, he'll do it," says Kevin Folta, a molecular biologist at the University of Florida in Gainesville, who led the woodland-strawberry project.

The plant world has many other challenging genomes to offer. The onion genome is massive, Folta says, and sugarcane has 12 copies of each chromosome. "Those will take special techniques," he says.

Every platform has benefits and drawbacks, and scientists must weigh the costs, sample-preparation time and sequencing-error rates for each. To sequence the woodland strawberry, for example, the scientists used a combination of three platforms.

But for polyploid genomes, short-read sequencing is almost a waste of time, says Clive Brown, chief technology officer at Oxford Nanopore. "You don't know where your short read comes from, which chromosome it is from," he says. "It's very hard to piece that together." He believes that the problem will be helped by instruments, including those in development in his company, that can generate long reads without the need for special sample preparation or complex assembly. The longer the reads, the easier the assembly, because the overlapping sequences will help researchers to determine which sequence belongs to which chromosome.

Fresh approaches were needed to crack the genome of the oil palm (*Elaeis guineensis*), reported last month in *Nature*[6]. The effort was more than a decade in the making. Oil palm is an important source of food, fuel and jobs in southeast Asia, and the industry is under pressure to produce it sustainably and avoid increased rainforest logging, says study co-leader Ravigadevi Sambanthamurthi, director of the advanced biotechnology and breeding centre at the Malaysian Palm Oil Board in Kajang, which works with the country's oil-palm industry.

With millions of repeats distributed throughout the plant's genome, short reads could fit in many possible spots in the assembled DNA sequence. "It is as if you were assembling a jigsaw puzzle in which most of the pieces are identical," says Robert Martienssen, a geneticist at Cold Spring Harbor Laboratory in New York, who co-led the project with Sambanthamurthi.

Classic sequencing methods were too laborious and expensive for the oil-palm project. So Martienssen suggested applying a technique based on a finding he had made in 1998 — that repeats in plant genomes can be distinguished from genes because the cytosine bases in the repeats usually carry methyl groups. Before fragments are sent to the sequencer, they are treated with enzymes that digest methylated DNA and thereby remove the repeats from the samples.

To complete the oil-palm project, the scientists applied this methylation-filtration technique and then sequenced the DNA regions housing genes. The technique has now been commercialized through Orion Genomics, a company based in St Louis, Missouri, which Martienssen co-founded.

The researchers used a high-throughput sequencer made by 454 Life Sciences, a company owned by Roche and based in Branford, Connecticut, that generates short reads from longer, filtered fragments. In preparing the samples, the researchers used bacteria to amplify DNA in large chunks on bacterial artificial chromosomes — an approach also used in the Human Genome Project — to pin down hard-to-map regions by retaining them next to genes with known positions to act as signposts.

Assembly of the oil-palm genome called for extensive computational resources, which crashed multiple times, the researchers say. But now, with the genome in hand, they have located a gene that encodes the shell of the palm fruit, knowledge they hope to harness to increase the plant's yield.

Sambanthamurthi says that when the researchers finally pinned down the shell gene, they popped a bottle of champagne, then celebrated with a traditional Malaysian meal served on a banana leaf.

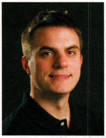
THE LONG AND THE SHORT

Bacterial genomes are smaller and less complex than those of plants and other multicellular organisms, but they, too, have regions that are tough to sequence. For example, *Bordetella pertussis*, which causes whooping cough, has hundreds of insertion sequence elements — stretches of mobile DNA inserted into various locations in the genome — each more than 1 kilobase long. Proponents of long-read technology say that spanning these regions with long reads will deliver sequencing efficiency gains.

Korlach points out that it took a team of more than 50 scientists to solve the bacterium's complete genome[7]. But long-read technology can make assembly of highly repetitive genomes faster and easier, he says. He says that he and scientists in the Netherlands were able to assemble nine whooping-cough bacterial strains in one month.

Whether a read is classified as 'long' or 'short' is in great flux. Two years ago, scientists might have said that a long read was 1 kilobase, Korlach says. "Now [Pacific Biosciences] customers are generating an average of 5,000 bases, with some reads longer than 20,000 bases — and we are working to deliver even more than that." Ultimately, a 'long read' will be as long as is needed to sequence a given genome, he says.

Korlach knows that some scientists say his company's sequencers are pricey, but he says that the newer versions have seen a significant drop in price and an increase in throughput. He says that the question of price is often raised "in the context of pure cost per sequenced base". And, he adds, if a certain sequencing technology is the only one that will work to solve a medically important question, "then there is no price tag that can be put on this medically relevant information".



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Titus Brown likens high-throughput sequencing to piecing together 1,000 shredded copies of a novel.

Last year, researchers collaborating with Pacific Biosciences used the company's sequencer to distinguish the repetitive genomic region involved in fragile X syndrome, a developmental disorder that is caused by repeats in a particular region on the X chromosome, and that worsens in severity with higher numbers of repeats[8].

As technology developers get closer to instruments that produce longer reads, scientists will need longer DNA fragments at the beginning of their sequencing experiments. Several companies focus on helping researchers to prepare DNA fragments for sequencing. For example, Sage Science, based in Beverly, Massachusetts, has a platform that uses pulsed-field electrophoresis to select and sort DNA fragments of sizes ranging from 50 base pairs to 50,000 base pairs. In May, the company began marketing its instrument to accompany the Pacific Biosciences sequencing platform.

Steve Siembieda, who is responsible for business development at Advanced Analytical Technologies in Ames, Iowa, says that his company sees the trend towards longer reads as writing on the wall. The company has licensed patents from Iowa State University, also in Ames, to develop an instrument to assess the integrity, fragment length and concentration of DNA samples.

With this instrument, an electric field is applied to a tiny amount of DNA so that it is pulled into a long, hair-thin capillary tube containing a gel with a fluorescent dye that binds to DNA molecules. As the DNA fragments move through the gel, they separate according to size. "Small molecules move fast, big molecules move slowly," Siembieda says. As the molecules pass by a window in the capillary, a flash of light excites the dye and a camera records the DNA fragment length (see 'Bits and pieces').

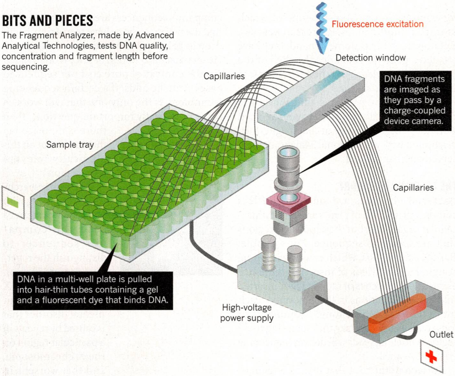
The instrument's readout tells scientists whether the size distribution of the DNA fragments is in the range needed for a given sequencing platform and whether the DNA

ROBERT MARTIENSSEN/CSHL

Oil palm has a complex and repeat-riddled genome that took more than ten years to sequence.

BITS AND PIECES

The Fragment Analyzer, made by Advanced Analytical Technologies, tests DNA quality, concentration and fragment length before sequencing.


Fluorescence excitation
Detection window
Capillaries
DNA fragments are imaged as they pass by a charge-coupled device camera.
Sample tray
Capillaries
DNA in a multi-well plate is pulled into hair-thin tubes containing a gel and a fluorescent dye that binds DNA.
High-voltage power supply
Outlet

ADVANCED ANALYTICAL TECHNOLOGIES

has the right concentration. Siembieda says that skipping these measurements can be the wrong experimental shortcut — if the concentration or fragment size is off, "a sequencer may run for nine days, it will cost them thousands of dollars, plus all the time wasted to not make sure they have the appropriate material". The instrument will possibly be used in developing the Moleculo process, but negotiations between the two companies are still under way.

Technology development at Advanced Analytical is focusing increasingly on long DNA fragments, which are challenging to resolve, Siembieda says. One solution is to customize gels for different applications. At present, the company's instrument can resolve lengths of up to 20 kilobases and the company is working on resolving longer fragments, he says.

ASSEMBLY REQUIRED

Scientists are applying many methods and tricks to create longer fragments. "Unfortunately, these technology tricks create erroneous data at points, so now you're stuck with some data that may be wrong," says Michigan State's Titus Brown. He was part of an effort, published in April[9], to sequence the lamprey (*Petromyzon marinus*) genome, one-third of which is covered by long repeats. Obtaining an assembly even with Sanger sequencing, which generates 1-kilobase reads, was difficult, he says. In addition, the lamprey genome has many GC-rich regions. The team used several types of software to assemble the complete DNA sequence.

In July, scientists published a comparison of software programs used to assemble sequence reads[10]. The researchers found that different assemblers give different results — even when fed the same sequence reads. Brown says that biologists should never forget that assemblies are not certainties. Every new sequencing technology — from how the DNA sample is prepared to the sequencing chemistry — has the potential for error and bias. "If you have short reads, or bad biology, you're going to have a very hard time getting a good assembly, even in theory," he says.

Ideally, a genome assembly should deliver end-to-end chromosomal sequences, says Shendure. What worries him more than the discordance among assemblers in the comparison study is that all of the assemblies were very fragmented. "That's not a fault of the assemblers, that's a fault of the data that we're putting into the assemblers and the fact that we're not capturing contiguity at these longer scales," he says. "The algorithms can only make do with the ingredients that they are provided by the technologies."

Brown is hopeful about the potential impact of longer-read technology. If Pacific Biosciences or Oxford Nanopore "deliver on many inexpensive long reads — more than 10 kilobases, I'd say — regardless of accuracy, you would end up revolutionizing the genome-assembly field, because it would give you so much more information to work with", he says. However, he adds, assembly software has to be compatible with each sequencing method. "So we're continually playing catch up, where new sequencing technologies lead to new sequence-analysis approaches a year or three later."

Eichler agrees that sequencing and assembly must continue to improve. Read lengths longer than 200 kilobases and with 99.9% accuracy rates will be needed to unpick repeats and other complications, he says. He says that the Pacific Biosciences instrument and what he knows of Moleculo "fall short of this, but are on the right track". All read-length requirements depend on the genome and complexity, he adds. For many bacterial genomes, current read lengths and accuracy are already sufficient, he says.

THE NEXT TELESCOPE

Oxford Nanopore plans to launch its new sequencing technology in the near future, but no date has been given. The technology expands on findings by researchers at Harvard University in Cambridge, Massachusetts, the University of Oxford, UK, and the University of California in Santa Cruz to harness the abilities of pore-forming proteins for DNA-sequencing devices.

One of the weaknesses of current high-throughput sequencing technology is amplification chemistry, says Oxford Nanopore's Clive Brown. Although DNA is made up of four bases, it is possible that more than those canonical four — such as bases that are methylated — should be detected, he says.

And in some sections of genomes, bases are naturally missing. But current sequencers do not capture such variations — instead, says Brown, they produce the equivalent of a four-colour photocopy of a picture with many more colours. "A lot of the detail is lost immediately, as soon as you make a four-colour copy," he says. Ideally, "you take a chromosome and run it through the sequencer. You can't quite do that yet." He, too, says that the next crucial phase of sequencing technology will be about long reads.

Brown says that to his mind, sequencers are just opening the door to characterizing the genome. People can get "very cosy about what they can see", with scientific instruments, he says. He likens today's sequencers to the first telescopes, which offered a view of the Moon's features and exploration of the visible spectrum. "It gets you a long way, you can count the stars, see the planets," he says. But the telescope does not show other celestial phenomena — such as dark matter or galactic movement.

Like astronomers with their telescopes, genome researchers will get a clearer picture of the genome as the sequencing technologies improve, he says. And, inspired by that picture, they will strive to see even more. ■

Vivien Marx *is technology editor for* Nature *and* Nature Methods.

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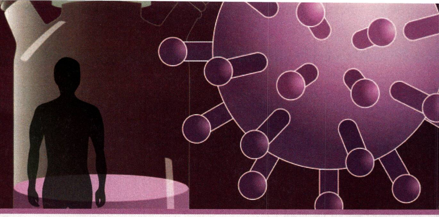

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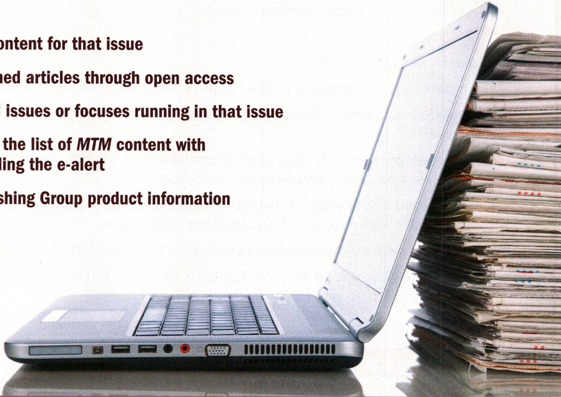

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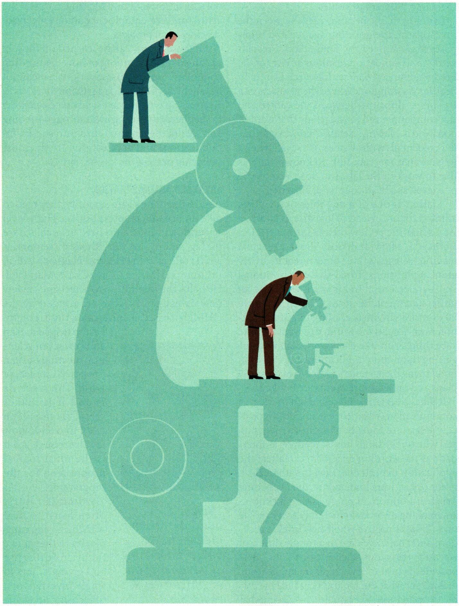

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ADMINISTRATION

A watchful eye on grant funding

Researchers disillusioned with the lab and eager to engage their soft skills can find promise in scientific administration.

BY PAUL SMAGLIK

Most scientists worry about how to secure grants. But a select few make a career out of managing those grants to ensure that the money is well spent. This is the job of the scientific administrator.

Broadly speaking, scientific administration involves the awarding, spending and tracking of funding at the grant, programme or policy level. Grant-level administrators assign or manage funds given to individual investigators. Programme administrators look after the needs of multi-investigator or multi-institution projects. And policy administrators oversee funding for entire departments, institutions or even university systems.

Jobs at all levels exist at universities, federal agencies and foundations, and scientific administrators often flip between these worlds at different stages of their careers — perhaps awarding grants at institutions such as the US National Institutes of Health (NIH) or UK biomedical charity the Wellcome Trust at one time, and managing funds at universities at another.

Scientific administrators have a crucial role in the research process, says Ginny Cox Delaney, an organizational consultant in the Oakland Office of the President of the University of California system, which administers ten research universities and five medical centres. "For me, science administration means the value-added role of advancing an organization's research goal, besides doing the actual science," she says.

HUMBLE BEGINNINGS

Almost no one sets out to be a scientific administrator. Instead, they realize at some point that they would rather support the scientific process away from the bench than at it.

When Elizabeth Prescott started her PhD, she had trouble visualizing where she would be in five years. After graduating, she deliberately chose a postdoc in the lab of a young investigator, at Yale University in New Haven, Connecticut. "I got a first-hand view of what it looked like to be a junior faculty member," says Prescott, noting the long hours the job entailed. "I don't think I love this enough," she thought.

She was considering leaving her fellowship and searching for a job in industry when the director of the postdoctoral-affairs office at Yale told her about an upcoming position. The university was establishing numerous core facilities in fields including DNA sequencing and high-performance computing, and the ▶

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provost's office needed someone to act as a liaison. Apart from a requirement to monitor how effectively the facilities served the needs of the university's scientists, the position was not well defined. So when Prescott got the job, she had a bit of a baptism of fire. "I learned about non-profit accounting; I learned about grant compliance," she says. She also learned to evaluate investigators' needs, and then sort them into different priorities.

In doing so, she became a link between the investigator, the granting agency and other parts of the university. These days, Prescott works as a foundation relations adviser at the Fred Hutchinson Cancer Research Center in Seattle, Washington, where she also acts as a liaison — this time between the centre and the companies and foundations that want to donate to it. Having scientific knowledge gives her credentials with all sides, she says. "It helps to have an understanding, to know what it is like to work in the lab. You don't want to be perceived as a bean counter or a barrier."

'Liaison' is one of many roles of scientific administrators. 'Head of logistics' is another. As a scientific review officer for the NIH in Bethesda, Maryland, Shiv Prasad recruits qualified scientists three times a year to review grant applications. To say that organizational skills are essential in his work would be an understatement, he notes. "When you have 20 or more scientists coming to a study section from around the world, you want to make sure things run like clockwork. That means getting them their applications to review on time, giving them enough notice to make travel plans and making sure rooms are booked."

It takes a certain kind of person to excel in administration — someone who enjoys repeatedly arranging and following up on meetings, and who understands how something as mundane as the wording on a form can affect the application experience, says Jonathan Best, grant operations manager at the Wellcome Trust in London. "I enjoy taking a process and making it work as efficiently as it can," he says. "It takes me to a whole new level of geekdom and challenges me in a totally different way to my previous scientific positions." Best provides help at every step, from application, making sure that the applicant has filed the correct information, to evaluation — seeing that members of the review committee get the applications in time. Currently his office is updating forms and revising deadlines to make the process less onerous for the applicant.

Administering grants also involves monitoring their success and implementation — activities that require attention to detail and a willingness to police recipients. After a grant has been awarded, Best helps to monitor how effective it was in advancing the investigator's field. He examines metrics such as publications and citations, as well as less tangible outcomes, including whether recipients have led effectively in their fields rather than following trends.

An ability to deal with deadlines is essential, says Diane McFadden, associate director of the Northeast Biodefense Center at Columbia University in New York — an NIH centre of excellence that involves 28 investigators and 3 postdocs at 12 institutions. With so many different components to manage, deadlines "are constantly hitting you", she says. And attention to detail is paramount: if someone does not fill out a form correctly and McFadden misses the error, funds or equipment could be delayed, slowing down projects.

All these things can be learned on the job, as can an understanding of how the funding and follow-up processes work. What cannot be taught, says Prasad, is a passion for science. Keeping up with the latest advances, helping to shepherd promising ideas through funding and seeing the research turn into successful publications and applications are the most satisfying parts of the job, he says.

ADVANCED ADMINISTRATION

As administrators gain seniority, they tend to take on broader responsibilities. High-level scientific administrators need to be able to see the big picture, says Carl Rhodes, a senior scientific officer at the Howard Hughes Medical Institute (HHMI) in Chevy Chase, Maryland. He helps the institution to select and review new investigators, who are funded by the HHMI but based at universities. Until recently, he also helped to plan and run seven scientific meetings a year. "You need to decide what the agenda is and if it makes sense," he says. "You need to decide how to advertise it, what is the most logical progression for the programme."

ROBERT DURELL/COPE

"Science administration means the value-added role of advancing an organization's research goal."
Ginny Cox Delaney

Administrators often learn to deal with increasing complexity as they move from monitoring and giving grants to single investigators, to focusing on whole programmes, departments or agencies. Rhodes started his teaching and administration career at Stanford University in California, where he focused on "things other faculty didn't want to do", he says, such as coordinating teaching assistants, refining the curriculum and dealing with the occasional disciplinary problem. He viewed himself as a service provider. "I made myself useful," he says.

In that role, Rhodes developed key skills and insights that would serve him well in a later job as a grants officer at the NIH, where he managed a review panel that evaluated joint PhD–medical doctorate programmes. It was also useful later on: sifting through 1,000 graduate-school applications at a time at Stanford was at least as difficult as triaging hundreds of new-investigator applications at the HHMI, he says. He now oversees scientific advisory panels that evaluate the strengths and weaknesses of each investigator's application and the contributions that they would make to their fields. The review officer combines the panel's comments into a single document — and all the documents go through Rhodes for final approval.

The ability to understand, create and evaluate budgets is essential to scientific administrators — especially as they move up the career ladder. McFadden has become adept at budgeting to make sure that her investigators' needs are met. "I've learned a tremendous amount about building a budget and how to do that with fairness," she says. Some projects deserve extra attention — and providing that, without belittling or alienating other projects in the consortium, can be a delicate process.

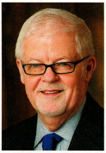
Having concrete goals — while keeping in mind a broader picture — is another hallmark of success in upper policy-level administration, says Sue Rosser, provost and vice-president for academic affairs at San Francisco State University in California. "The higher up you get in administration, the more emphasis there is on having a goal and getting things done and on time," she adds. Rosser works to make sure that the myriad meetings that populate her days are not empty exercises, but produce something tangible. "I like to have agendas, outcomes, follow-ups, results," she says. For example, last year her institution decided not to renew funding for a multiyear, multimillion-dollar project that did not fit the university's mission. More than a year before the programme was due to end, Rosser and her team began to have meetings about the closure, with detailed timelines to complete the programme's activities and ensure a smooth transition to an interim grantee. Rosser, like Rhodes, has a service mindset — but she is not so closely connected to the people she helps.

"I am trying to help the faculty under me succeed," she says. "And I am not even directly doing that, I am helping deans help chairs help faculty."

A WAY IN

Most people who move from a research career to scientific administration do so when they realize that lab work does not meet their life and career goals. There is no obvious, well-worn career path that reliably culminates in an administrative post, but there are ways to get a foothold — and to find out whether it is the right route. When Cox Delaney recognized that she did not have "the golden hands" needed to succeed in the lab, she got a public-policy fellowship from the American Association for the Advancement of Science in Washington DC. That had her working on science-policy cooperation between the United States and western Europe, which helped her to develop her interpersonal communication skills. A subsequent job at the Alfred P. Sloan Foundation, a non-profit granting organization based in New York, taught her about scientific funding.

Cox Delaney says that graduate students and postdocs who think they might be interested in administration can gather experience by running symposia, planning talks, dealing with caterers or booking speakers and venues for conferences. "There are a lot of opportunities to step up into leadership roles," she says. "That will give you a sense of whether you like the organizing piece."



JAMES KEGLEY

"You need to decide what is the most logical progression for the programme."
Carl Rhodes

Even pulling authors together for a paper or coordinating a simple event such as a journal club can help, if the organizers can observe the people they are working with and find out what motivates them, or can build new skills, says Cox Delaney. She is currently working with all the University of California campuses to find ways to share and save on administrative costs, so that they can pass the savings on to research and education.

Advancing in administration often means nurturing a skill set that goes well beyond research. Cox Delaney puts people who go into scientific administration into two categories: people with a strong understanding of science and good interpersonal skills; and people adept at accounting and funding. Administrators who have both sets of traits are relatively rare — and they are the ones who tend to rise to the highest levels, she says.

Many universities — especially publicly funded, research-based ones — recruit their leading managers from a pool of scientists, who tend to be detail-oriented and adept at handling complexity, and researchers with a particular appetite and aptitude for managing budgets and people will have multiple career options in administration. The path to broader responsibilities, or even a university presidency, could begin with shepherding a single grant. ■

Paul Smaglik *is a freelance writer based in Milwaukee, Wisconsin.*

RESEARCH

Biology in space

NASA is launching an open-ended research programme to investigate how human and other tissue reacts to time spent in space. The geneLAB project will begin seeking grant applications by autumn 2014, says D. Marshall Porterfield, director of space life and physical sciences research at NASA in Washington DC. It will award 'innovation exploration' grants of US$100,000 for one year; full grants will be for up to 5 years and worth a maximum of $500,000. The agency wants to send organisms including fruitflies and roundworms to the International Space Station to learn how spaceflight affects living tissue at the biomolecular and genetic level. Future grant recipients would also study bone loss and examine tissue from crew members to look for changes to their DNA that occurred while in space and after returning to Earth.

GENDER

Mothers' careers stalled

Attitudes about motherhood can impede women's career aspirations, even at companies that purport to have family-friendly policies, a study suggests (C. Herman *et al. Gender Work Organ.* **20,** 467–478; 2013). Women working in science, engineering and technology at multinational corporations in the Netherlands, France and Italy adopted potentially career-damaging tactics including, for example, avoiding big projects and disguising the need to leave early or come in late because of childcare obligations, the study found. Firms must take stock of how attitudes stymie women who are looking to advance, says study co-author Anne Laure Humbert, a gender researcher at the European Institute for Gender Equality in Vilnius.

FUNDING

Genius grant grows

Awards for the MacArthur Fellows Program, known as genius grants, will this year rise from US$500,000 to $625,000. The John D. and Catherine T. MacArthur Foundation in Chicago, Illinois, makes unrestricted 5-year grants to recipients chosen for their creativity, innovation and potential to shape the future. Spokesman Andrew Solomon says that the increase is partly a response to inflation and is the programme's fourth rise since it began in 1981. The 2013 fellows will be announced on 25 September.

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The Department of Molecular and Cell Biology (MCB) is seeking applications for four faculty positions in the areas listed below. We seek candidates with Ph. D. and/or M.D. degree who have demonstrated excellence, originality and productivity in research, and interest in undergraduate and graduate teaching. Ph.D. or equivalent is required by date of hire.

Applications should include a *curriculum vitae*; a list of publications; copies of three significant publications; a brief description of research accomplishments; and a statement of research objectives and teaching interests. We are particularly interested in candidates who will contribute to diversity and equal opportunity in higher education through their teaching, research, and service; candidates are invited to address this issue in their application. Applicants for the tenure level in the Biology of Cancer or Emerging & Neglected Diseases searches should include a list of five references with their application; letters of reference are not required at this time. Applicants for the Assistant professor level positions should arrange to have three letters of reference submitted online. All letters will be treated as confidential per University of California policy and California state law. Please refer potential referees, including when letters are provided via a third party (i.e., dossier service or career center), to the UC Berkeley statement of confidentiality (http://apo.chance.berkeley.edu/evalltr.html) prior to submitting their letters.

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We seek candidates interested in the mechanisms of cancer and treatment of this disease with a primary focus on human cancer or mammalian models. Areas of interest include (but are not limited to) the genetics and epigenetics of cancer in humans or mouse models, tumor-host interactions, tumor metastasis, tumor immunology, cancer genomics, and the use of mouse models to dissect mechanisms of tumor initiation and progression. The successful candidate will have the opportunity to provide leadership in the field of cancer biology at the university in the future. This position is open at any level (tenure-track or tenured). *Applications should be submitted online through https://aprecruit.berkeley.edu/apply/JPF00217. See above application details.*

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Group and Team Leader Opportunities

GENOME BIOLOGY

at EMBL Heidelberg, Germany

The Genome Biology Unit studies the molecular mechanisms by which genetic information is expressed and regulated to yield complex biological systems, and how genetic variation leads to phenotypic diversity. The Unit addresses questions at different scales, ranging from detailed mechanistic studies (using genetics, chemistry, biochemistry and microfluidics) to genome-wide studies (using functional genomic, proteomic and computational approaches). This powerful combination enables the unit to dissect and model complex processes going from genotype to phenotype.

This **Group Leader** position provides a very competitive package to support an independent research group in an excellent scientific and highly collaborative environment. We particularly encourage candidates applying a systems biology approach to understanding specific aspects of gene expression regulation, evolution, metabolomics, stem cell biology or genomic applications to health to apply, but welcome candidates with demonstrated excellence in any area of genome biology.

Further information about the position can be obtained from the Head of Unit, Eileen Furlong (**eileen.furlong@embl.de**).

Interviews are planned for 25, 26 and 27 November 2013.

COMPUTATIONAL BIOLOGY/ BIOINFORMATICS

at EMBL Heidelberg, Germany

Bioinformatics activities are integral to the interdisciplinary research activities at EMBL and thus we seek to recruit an outstanding **Group or Team Leader**, working in the broad area of computational biology and bioinformatics, who would utilise the highly interdisciplinary and collaborative environment at EMBL Heidelberg.

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We are open to applications from candidates who wish to focus entirely on their own research projects and, because there is an increasing need to integrate and streamline the multiple computational approaches followed within EMBL, to candidates who wish to function at the interface between research and service activities.

For further information about this position please contact **recruitment@embl.org**.

Interviews are planned for 9, 10 and 11 December 2013.

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at EMBL Grenoble, France

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Further information about the position can be obtained from the Head of Grenoble Outstation, Stephen Cusack (**cusack@embl.fr**).

Interviews are planned for 2, 3 and 4 December 2013.

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Assistant Professor Positions Available at Princeton University

The Lewis-Sigler Institute for Integrative Genomics at Princeton University invites applications for tenure-track faculty positions at the Assistant Professor level; in exceptional cases, more senior appointments may be considered. We are seeking outstanding scientists with strong interest and experience in quantitative, systems-level approaches to understanding any area in modern experimental molecular biology, chemistry, or evolutionary biology. Appointments at the Lewis-Sigler Institute are made jointly with an appropriate home department. A strong record of experimental work and quantitative analysis is essential. The successful candidate will have research laboratories at the Institute, and teaching responsibilities (both graduate and undergraduate) will be shared between the Institute and the home department.

The Lewis-Sigler Institute for Integrative Genomics, housed in the Carl Icahn Laboratory at Princeton University, was established to innovate in research and teaching at the interface of modern biology and the more quantitative sciences. The Institute provides significant support to its faculty through heavy investment in state-of-the-art research infrastructure, an NIH-funded graduate training program, and a high level of administrative support as well.

Essential Qualifications

All applicants must have a Ph.D., M.D., or equivalent degree. In addition, applicants must have a very strong record of research productivity, demonstrate the ability to develop a rigorous research program, and be committed to teaching at both the undergraduate and graduate levels.

How to Apply

Applications must be submitted online at: http://jobs.princeton.edu/applicants/Central?quickFind=64204, requisition # 1300612 and should include a cover letter, curriculum vitae, a two-page research description, as well as contact information for three references. Applications will be reviewed beginning on October 15, 2013.

Princeton University is an equal opportunity employer and complies with applicable EEO and affirmative action regulations.

NW240432R

Assistant Professor

Department of Biological Chemistry and Molecular Pharmacology

Harvard Medical School

The Department of Biological Chemistry and Molecular Pharmacology at Harvard Medical School invites applicants for a tenure-track faculty position at the rank of Assistant Professor. We are seeking individuals with a demonstrated potential for imaginative research and who propose to work on exciting problems in any area of chemical biology, structural biology, or single-molecule biochemistry. The successful candidate will be expected to direct innovative and independent research and participate in the teaching of graduate and/or medical students. Our highly interactive environment provides the opportunity to engage and collaborate with other dedicated researchers both within the Department and throughout the diverse Harvard research community. Significant scholarly and scientific resources will be made available for this appointment. For further information about our Department, please see our Web Page: **http://bcmp.med.harvard.edu**.

Applicants should submit electronic copies of their curriculum vitae, a description of research accomplishments and future research interests (three pages maximum), and ask at least three references to provide letters of recommendation.

These materials should be submitted using the following link:
https://academicpositions.harvard.edu/postings/4973
Applications will be considered starting November 1, 2013.

Harvard Medical School is an Equal Opportunity/Affirmative Action employer. We are actively committed to increasing the diversity of our faculty. Women and members of underrepresented minority groups are therefore strongly encouraged to apply.

HARVARD
MEDICAL SCHOOL

NW240489R

Temple University School of Medicine (TUSM) / Center for Metabolic Research Center (CMDR) seeks to recruit basic science researcher for tenure-tracked department faculty positions at the Assistant or Associate Professor levels, depending on qualifications.

The applicant will have M.D., Ph.D., or M.D./Ph.D. degrees, have completed rigorous training and have the ability to develop an independent extramurally funded laboratory. The ideal candidate will have expertise and have applied modern approaches in studying biochemistry and molecular biology basis of metabolic disease, including hyperlipidemia, hyperhomocysteinemia, hyperglycermia, obesity, diabetes, uremia and metabolic syndrome.

Candidates are asked to send their applications (including curriculum vitae, a concise research summary, and names and contact information for three references) to: Dr. Hong Wang, M.D, Ph.D, EMBA, Director, CMDR, Temple University School of Medicine, 3500 North Broad Street, Philadelphia, PA 19140 (hong.wang@temple.edu)

Temple University is an affirmative action/equal opportunity employer and strongly encourages applications from women and minorities.

NW240540R

FACULTY POSITION IN BIOLOGICAL SCIENCES AT VANDERBILT UNIVERSITY

The Department of Biological Sciences at Vanderbilt University seeks candidates to fill a tenure-track, assistant professor faculty position. We desire candidates whose research complements existing areas of strength within the department (**http://as.vanderbilt.edu/biosci**). The scope of the search will reflect the full breadth of the department; we are particularly interested in candidates working at the intersection of development, evolution and molecular genetics. The central criteria for the position are excellence in research and the ability to teach undergraduate and graduate students with a high level of effectiveness. Women and under-represented minority candidates are especially encouraged to apply.

Applicants should send a single PDF containing a letter of application, curriculum vitae, statement of current and future research interests, and evidence of teaching effectiveness to **nikki.moore@vanderbilt.edu.**

Applicants should arrange for three letters of recommendation to be sent to the same address. Review of applicants will begin November 1, 2013, and will continue until the position has been filled.

Vanderbilt University is an Affirmative Action / Equal Opportunity Employer.

NW240704R

METABOLOMICS FACULTY POSITION BIOLOGICAL CHEMISTRY & PEDIATRICS

The Johns Hopkins University
School of Medicine

The Departments of Biological Chemistry (BC) and Pediatrics (PD) at The Johns Hopkins University School of Medicine invite applications for a tenure-track faculty position at any rank. BC & PD are jointly seeking candidates with an outstanding record in the area of metabolomics, and a commitment to excellence in research and teaching who will conduct pioneering independent research and also serve as the faculty director of the Johns Hopkins Metabolomics Core Laboratory. Applicants should submit (preferably as a single PDF file) a curriculum vitae, a list of publications, and a summary of research accomplishments and future plans by **December 15, 2013.** Electronic files should be sent to **metabolomics@jhmi.edu.** Applicants should also request that 3 letters of recommendation be sent electronically (preferred) or mailed to the address below:

Gerald W. Hart, Ph.D.
Committee Chair
c/o Ms. Darlene Sutton
Department of Biological Chemistry
The Johns Hopkins University
School of Medicine
725 North Wolfe Street
Baltimore, MD 21205-2185

Equal Opportunity/Affirmative Action Employer

NW239441R

JOHNS HOPKINS
UNIVERSITY

Bloomberg Distinguished Professor of Molecular and Cellular Neuroscience
The Johns Hopkins University

A Bloomberg Distinguished Professorship in Molecular and Cellular Neuroscience, with a focus on brain development and function, is available at the Johns Hopkins University. This position is one of 50 new Bloomberg Distinguished Professorships designated for outstanding scholars who carry out interdisciplinary research and teaching in areas identified for significant growth at the University. The current position is linked to growth in the University-wide Science of Learning Initiative. The position will include joint tenure in the School of Medicine and the Krieger School of Arts and Sciences, crossing the Departments of Neuroscience, Psychological Brain Science, and/or Biology. The holder of this Bloomberg Distinguished Professorship will participate in research and teaching in both the School of Medicine and the Krieger School of Arts and Sciences. Applicants should possess distinguished records of achievement in both research in molecular and cellular neuroscience and the teaching of undergraduate, graduate, and medical students compatible with appointment to tenure at the Johns Hopkins University. Investigators interested in the molecular and cellular basis of nervous system development, synaptic plasticity, circuitry and function are invited to apply. Please submit a PDF file containing curriculum vitae to the email address below. The deadline for application is November 1st, 2013.

The Johns Hopkins University is committed to enhancing the diversity of its faculty and encourages applications from women and minorities. Please submit a PDF file containing curriculum vitae to the email address below.

Richard L. Huganir
Michela Gallagher
Co-Chairs Search Committee
The Johns Hopkins University, School of Medicine,
725 North Wolfe Street, Hunterian 1009A, Baltimore, Maryland 21205
HopkinsNeuroBDPSearch@jhmi.edu

An EEO/AA employer

NW240175R

University of Massachusetts
UMASS Medical School

Faculty Position in Innate Immunity
University of Massachusetts Medical School

UMMS is located within a 67-acre integrated health science campus, encompassing the School of Medicine, Graduate School of Biomedical Sciences, and Graduate School of Nursing, located in the picturesque Worcester Hills/Lake Quinsigamond region of central Massachusetts. The Division of Infectious Diseases and Immunology (**http://www.umassmed.edu/infdis/index.aspx**) has a major focus on innate immunity, autoimmunity and host-microbe interactions, utilizing human, murine and invertebrate models and coordinates research activities of the recently formed Program for Innate Immunity.

We are currently recruiting for a full-time tenure-track faculty position. Appointed rank will be commensurate with experience. Faculty at UMMS engage in teaching, research and service, and successful candidates will be expected to direct an externally funded independent research program as well as participate in graduate education. Laboratory space and extensive core facilities are available in the recently constructed Lazare Research Building. Candidates must have a Ph.D. and/or M.D. (or equivalents), and a record of significant research accomplishments in any area of innate immunity. Highest priority will be given to investigators with outstanding research programs that complement our current strengths.

Successful candidates will also demonstrate strong communication skills and an interest in collaborative research endeavors. Review of applications will begin on September 15th, 2013 and will continue until the position is filled.

Applicants should upload a cover letter, CV, research statement and publication list to **https://academicjobsonline.org/ajo/jobs/2947**.

To expedite the review process, applicants should also invite three individuals who are familiar with their work and potential for success to upload recommendation letters to **http://www.academicjobsonline.org**.

Please refer any questions regarding the search to: Neal Silverman, Search Committee Chair and Professor of Medicine, **neal.silverman@umassmed.edu**

As an equal opportunity and affirmative action employer, UMMS recognizes the power of a diverse community and encourages applications from individuals with varied experiences, perspectives and backgrounds.

NW240026R

Yale SCHOOL OF FORESTRY & ENVIRONMENTAL STUDIES

FACULTY POSITION
PLANT PHYSIOLOGICAL ECOLOGY

The School of Forestry and Environmental Studies at Yale University invites applications for an appointment at the Full, Associate, or Assistant Professor level in Plant Physiological Ecology with a focus on woody plants. Preference will be given to applicants working at the level of whole organisms and specializing in the interaction of trees and forests with their environment.

The successful candidate will have a doctoral degree in a related field, as well as an excellent research program, grounded in plant physiology, structure, and function. Research should address problems of broad importance, such as the impacts of climate change or management practices at multiple scales, from the level of individuals and populations to large-scale ecosystems and biomes. We are especially interested in candidates with the capacity to collaborate with other faculty in a strongly interdisciplinary School, as well as with faculty in the Departments of Ecology and Evolutionary Biology, Geology and Geophysics, and Molecular, Cell and Developmental Biology.

Applicants will be expected to teach classes to PhD, Masters and undergraduate students, and to develop an internationally-recognized research program that involves graduate students, including through fieldwork. The ability to teach and conduct research in both temperate and tropical systems would be an asset.

Please submit materials by Friday, September 27, 2013 for full consideration.

To apply, visit **https://academicjobsonline.org/ajo/jobs/2686**. For questions, email fesdeansoffice@yale.edu. Salary and rank will be commensurate with experience.

Yale University is an affirmative action/equal opportunity employer; applications from women and minorities are encouraged.

http://environment.yale.edu

NW239866R

DIRECTOR, JOINT CENTER FOR EARTH SYSTEMS TECHNOLOGY

University of Maryland, Baltimore County

The University of Maryland, Baltimore County (UMBC) seeks a Director for its Joint Center for Earth Systems Technology (JCET), who will also qualify for appointment as a tenured full professor in an academic department at UMBC. JCET was established in 1995 and is funded primarily by a cooperative agreement with the NASA Goddard Space Flight Center (GSFC). JCET has a research staff of approximately 45 scientists and faculty, many of who collaborate with scientists at GSFC and other entities in research relating to the observation, analysis and modeling of physical processes within the Earth-Sun system, and to global climate change issues on the Earth. More information can be seen at **http://jcet.umbc.edu/.**

The Director provides the overall scientific vision for JCET, and is the center's principal interface to both GSFC and to the UMBC academic community. The Director is responsible for the overall management of the Center, and for the scientific quality and integrity of the research performed by the Center's research staff, who are located at both GSFC and UMBC. Up to full academic year salary is available and the Director will be expected to spend a significant amount of time on research and teaching at UMBC. The Director reports to the Vice President for Research.

The Director should possess a broad scientific and technical knowledge of Earth, atmospheric and oceanic sciences and should possess a distinguished research portfolio in one of these fields. A Ph.D. is required.

UMBC is a dynamic and fast growing institution that has been named by US News and World Report as the top up and coming university for four consecutive years. UMBC is classified by the Carnegie Foundation as a High Research Activity research university. It is located in the Baltimore-Washington corridor close to BWI airport and many federal laboratories including GSFC. The university was founded in 1966 and has grown to approximately 14,000 students including approximately 3,000 graduate students.

Applications including a letter of interest, a current c.v., and the names, addresses, telephone numbers and email addresses for at least three references, should be sent electronically to **allison@umbc.edu.** In order to receive full consideration applications should be sent before October 21, 2013. The search will remain open until the position is filled. For further information and questions contact Dr. Geoff Summers at **gsummers@umbc.edu.** UMBC is an Equal Opportunity/Affirmative Action Employer.

NW240363R

Faculty Position in Virology

Department of Microbiology-Immunology

Northwestern University Feinberg School of Medicine

A tenure-track position is open for a full-time faculty researcher at the level of Assistant/Associate Professor in Virology (PhD, MD/PhD or MD). Areas of particular interest include virus-cell interaction, pathogenesis, assembly, oncogenesis, immune evasion, and innate response to viral infection. Areas of study relevant to human disease are of particular interest.

All applicants should have postdoctoral research experience with substantial peer-reviewed publications that document research productivity and the ability to perform cutting edge research. A demonstrated ability to secure external funding is recommended. Candidates should have an interest in teaching graduate and medical students. Starting date and salary are negotiable. Application materials will be reviewed as received. Please send a complete CV and the name and contact information of at least three references by email to: **juniorvirologist@northwestern.edu.**

Northwestern University is an Affirmative Action, Equal Opportunity Employer. Women and minorities are encouraged to apply. Hiring is contingent upon eligibility to work in the United States.

NW240209R

Fall 2013

Regulatory Systems Biology – Faculty Position

The California Institute of Technology invites applications for a tenure-track assistant professor position in the Division of Biology and Biological Engineering. We are interested in candidates who will approach gene-centered questions in the fundamental biology of multicellular organisms from a complex system perspective. Relevant research areas include but are not limited to gene network and circuit design problems in developmental biology, evolution of gene regulatory networks, and design principles in gene regulatory circuits.

Candidates with strong commitments to research and teaching excellence are encouraged to apply. Initial appointments at the assistant professor level are for four years, and they are contingent upon completion of the Ph.D. degree.

Please submit on-line application at: http://biology.caltech.edu/Positions, and include a brief cover letter; curriculum vitae; relevant publications, a description of proposed research; and a statement of teaching interests. Applicants should arrange to have four reference letters uploaded.

Application review will commence immediately and continue until Jan 1, 2014.

The California Institute of Technology is an Equal Opportunity/Affirmative Action Employer. Women, minorities, veterans, and disabled persons are encouraged to apply.

NW240240R

FACULTY POSITIONS AVAILABLE

in the Department of Cell and Molecular Biology

Feinberg School of Medicine, Northwestern University

The Department of Cell and Molecular Biology at the Feinberg School of Medicine, Northwestern University, seeks to recruit several full-time tenure-track faculty working in the fields of cell and molecular biology. Positions are available at all ranks from assistant to full professor. Applicants must hold a PhD or MD/PhD degree. Applications, including a cover letter, a curriculum vitae and a statement of research interests should be sent electronically as a single pdf document to **cmbsearch@northwestern.edu.** Candidates applying for an Assistant Professor position should provide three letters of recommendation which must be submitted electronically to the same e-mail address. Letters should be provided in pdf format and should include the name of the applicant in the subject line. Candidates for Associate or Full Professor should provide names and the contact information of three individuals willing to submit letters of recommendation on their behalf.

Northwestern University is an Affirmative Action/Equal Opportunity Employer. Hiring is contingent upon eligibility to work in the United States. Women and minorities are encouraged to apply.

NW240359R

Princeton University Department of Chemistry

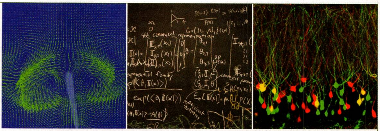
Assistant Professor - All Areas

The Department of Chemistry at Princeton University invites applications for a tenure-track assistant professor position in all areas of chemistry. We seek faculty members who will create a climate that embraces excellence and diversity, with a strong commitment to teaching and mentoring that will enhance the work of the department and attract and retain students of all races, nationalities and genders. We strongly encourage applications from members of all underrepresented groups.

Candidates are expected to have completed the Ph.D. in chemistry or a related field at the time of appointment. Applicants should submit a description of research interests, curriculum vitae, a list of publications, and contact information for three referees online at **jobs.princeton.edu/applicants/Central?quickFind=64207.** The search committee will begin review of applications on 10/17/2013 and will continue until the position is filled.

Princeton University is an Equal Opportunity employer and complies with applicable EEO and affirmative action regulations.

NW240395R



CALL FOR
Professors and Assistant Professors

IST Austria invites applications for **tenured** and **tenure-track leaders** of **independent research groups** in following fields: **Physics | Chemistry | Biology | Neuroscience | Earth Science | Mathematics | Computer Science | Interdisciplinary Areas**

The Institute is dedicated to basic research and graduate education in the natural and formal sciences. The successful candidates will receive a substantial annual research budget, are expected to apply for external research grants and to participate in the Graduate School.

Deadline for receiving Assistant Professor applications: November 15, 2013
Open call for Professor applications
Further information and online application: **www.ist.ac.at/professor-applications**

IST Austria values diversity and is committed to equality. Female researchers are encouraged to apply.

IST AUSTRIA
Institute of Science and Technology

W240345R

UT SOUTHWESTERN
MEDICAL CENTER

Assistant Professor in Human Genetics

The Center for Human Genetics (McDermott Center for Human Growth & Development) at UT Southwestern Medical Center **(http://www.utsouthwestern.edu/education/medical-school/departments/mcdermott-center/index.html)** invites applications for a tenure-track position of Assistant Professor. We are seeking individuals with innovative experimental research programs in human molecular genetics. Successful applicants will be expected to establish a vigorous independent research program and to teach students at the graduate level.

The individual should hold a graduate degree (MD, PhD or MD/PhD) and have completed a post-doctoral fellowship or be a candidate for the NIH Director's Early Independence Award. The appointment will include a competitive salary, attractive start-up package, excellent laboratory space in a dynamic research environment with access to the Center's state-of-the-art infrastructure for basic and clinical research related to human genetics, including family/patient collection, genetic variation, next-generation sequencing and bioinformatics cores. The faculty member will be jointly appointed to a basic science or clinical department. Applicants should submit their curriculum vitae containing a summary of past accomplishments, a statement of future objectives, and three professional references by email to:

Faculty Search Committee
c/o Susan Hayes, Dept. Administrator
McDermott Center for Human Growth & Development
UT Southwestern Medical Center at Dallas
5323 Harry Hines Boulevard
Dallas, Texas 75390-8591

Susan.Hayes@UTSouthwestern.edu

UT Southwestern Medical Center is an Affirmative Action/Equal Opportunity Employer. Women, minorities, veterans and individuals with disabilities are encouraged to apply.

NW240132R

VE RI TAS

HARVARD UNIVERSITY
Psychology / Center for Brain Science
Faculty Search

The Department of Psychology, in conjunction with the Center for Brain Science, seeks faculty applicants whose research is focused on neurobiological approaches to human brain function. Areas of interest include, but are not limited to, perception, sensory and motor function, decision-making, biological bases of psychiatric disease, connectomics, and theoretical neuroscience. The successful appointee will be expected to strengthen links between the Department and the Center, where research focuses on the structure and function of neural circuits and their relationship to behavior. The appointment is expected to begin on July 1, 2014. Candidates at all levels are encouraged to apply.

Candidates should have demonstrated excellence in both research and teaching. Teaching opportunities will include offerings at both undergraduate and graduate levels.

An appointment may be made at either the tenured or tenure-track (assistant or associate) levels.

Candidates for tenured positions should upload applications to:
http://academicpositions.harvard.edu/postings/4935.

Candidates for tenure-track positions should upload applications to:
http://academicpositions.harvard.edu/postings/4933.

Questions regarding this position can be addressed to **BrainSearch@wjh.harvard.edu.** The committee will begin considering completed applications on **November 15, 2013.**

Harvard University is an Affirmative Action/Equal Opportunity Employer, and we actively encourage applications from women and minority groups.

NW240190R

RUTGERS
NEWARK

The Center for Molecular & Behavioral Neuroscience at Rutgers University has **two** openings for tenure-track Assistant Professors in **SYSTEMS NEUROSCIENCE.** Applicants should hold a Ph.D. degree in Neuroscience or a related field and have completed at least two years of post-doctoral training.

Rutgers, The State University of New Jersey, is a leading national public research university and the state's preeminent, comprehensive public institution of higher education. Rutgers is dedicated to conducting research that breaks new ground; to teaching that meets the highest standards of excellence; and to providing services, solutions, and clinical care that help individuals and the local, national, and global communities where they live.

The Center for Molecular and Behavioral Neuroscience (CMBN) is located in Newark NJ, a short drive from New York City. CMBN is part of a large community of neuroscientists at Rutgers Newark, with additional researchers in the Departments of Biological Sciences and Psychology as well as in the Rutgers University New Jersey Medical School. Collectively, these researchers represent all levels of analysis in neuroscience (http://www.neuroscience.newark.rutgers.edu).

The ideal candidates for these two assistant professor positions ask fundamental hypothesis-driven questions about brain structure and function and are open to establishing collaborative research efforts within the Rutgers neuroscience community. Research areas of particular interest are sensory-motor integration, learning and memory, and emotional regulation. We are especially interested in candidates who use a range of experimental methods, including in vitro and in vivo electrophysiology in rodents or non-human primates, optogenetics, tract tracing and immunohistochemistry. The successful applicants will be expected to maintain an active externally-funded research program, to teach undergraduate and graduate courses in neuroscience, and to mentor Ph.D. students in our Behavioral and Neural Sciences program.

Applicants should apply as soon as possible, but no later than 10/15/2013. Rutgers is an Equal Opportunity/Affirmative Action Employer and has a strong commitment to diversity. Women and members of underrepresented minorities are especially encouraged to apply. Rutgers-Newark is noted for its exceptionally diverse student population.

To receive detailed instructions on how to apply, please send an email to systems@cmbn.rutgers.edu with the subject line 'How to apply'.

NW239703R

Department of Neurobiology
Duke University Medical Center

FACULTY APPOINTMENTS IN NEUROBIOLOGY
Duke University Medical Center
Department of Neurobiology

As part of its recruitment of up to 5 neurobiologists in the next 3 years, Duke University invites applications for tenure-track neuroscientists in the Department of Neurobiology. In the academic year 2013-14, we are searching for talented investigators for up to 2 faculty positions. Appointments may be at any level, with a preference for tenure-track Assistant Professors who would start their independent careers at Duke. Research programs may use approaches from molecular, and cellular to systems and computational neuroscience to ask questions about the development and function of the brain. Successful candidates will have track records of creativity and productivity, demonstrated potential for outstanding future achievements, and a desire to participate in an exciting, interactive, and growing community. They will be expected to mount a productive and innovative research program, to obtain outside funding, and to participate actively in graduate education. Candidates must have a Ph.D., M.D., or equivalent degree. Qualified women and minority candidates are especially encouraged to apply. For full consideration, applications should be submitted by November 1, 2013, although applications will be considered until the positions are filled. Applicants should submit a curriculum vitae, a brief summary of current and proposed research, reprints of 3 key publications, and 3 letters of recommendations to:

https://academicjobsonline.org/ajo/jobs/2936

Questions may be directed to Stephen Lisberger, Chair of the Department of Neurobiology, **LISBERGER@NEURO.DUKE.EDU**

Duke University is an Equal Opportunity Affirmative Action Employer.

NW240095R

FACULTY POSITIONS AT THE ROCKEFELLER UNIVERSITY

The Rockefeller University seeks exceptional, interactive, and creative scientists to join its faculty. We invite applications from outstanding candidates for tenure-track positions.

The University has a laboratory-based organizational structure that fosters interdisciplinary research. We encourage applications in the following areas:

- **Chemical & Structural Biology**
- **Genetics & Genomics**
- **Immunology, Virology & Microbiology**
- **Medical Sciences, Systems Physiology & Human Genetics**
- **Molecular Cell Biology**
- **Neurosciences & Behavior**
- **Organismal Biology, Evolution, Ethology & Ecology**
- **Physical, Mathematical & Computational Biology**
- **Stem Cells, Development, Regeneration & Aging**

Details about specific subjects of research can be found at **http://www.rockefeller.edu/facultysearch.**

The Rockefeller University provides strong support for the research work of its faculty. The positions offer competitive salary, benefits and start-up funds, renovated laboratory space, access to state-of-the art core facilities and extensive opportunities for collaboration both within the University and with neighboring institutions.

Applications are being accepted electronically through our **Online Application System** at **http://oas.rockefeller.edu**. Applicants should follow the online application procedure.

The deadline for application submission is October 11, 2013.

If you have questions regarding submitting an application, please contact our Administrator at **facultysearch@rockefeller.edu**.

The Rockefeller University is an Affirmative Action/ Equal Opportunity/VEVRAA Employer and solicits applications from women and under-represented minorities.

NW240366R

BE A PART OF THE CURE

Moffitt Cancer Center invites applications for a tenure-track faculty position in the Basic Sciences Division and the Program of Cancer Biology and Evolution. Appointment will be at the level of Assistant Member or Associate Member depending on candidate's qualifications and experience. Successful candidate will be part of a highly collaborative, multidisciplinary team focused on the molecular, cellular and environmental components of Darwinian dynamics in cancer biology and therapy.

Applicants should have excellent post-doctoral training as evidenced by outstanding publications and demonstrate the potential for obtaining extramural grants. In addition, candidates applying for appointment at Associate Member level should have sustained extramural funding.

For further details and to apply,
visit MOFFITT.org/Careers

CLOSER TO OUR PATIENTS.
CLOSER TO A CURE.®

MOFFITT
CANCER CENTER

12902 MAGNOLIA DRIVE, TAMPA, FL 33612 | 1-888-MOFFITT | MOFFITT.ORG

Moffitt Cancer Center provides a tobacco-free work environment. We are an equal opportunity, affirmative action employer and a drug-free workplace.

NW240348R

Faculty Positions in Gene Regulation and Genomics

The University of Texas Southwestern Medical Center in Dallas, the **Cecil H. and Ida Green Center for Reproductive Biology Sciences** and the Division of Basic Reproductive Biology Research in the Department of Obstetrics and Gynecology is recruiting. We invite applications from outstanding candidates for three tenure-track assistant professor positions in signaling, gene regulation, and genome function, especially in the areas of chromatin and transcription, epigenetics, nuclear endpoints of cellular signaling pathways, nuclear receptors, RNA biology, genome organization and evolution, and DNA replication and repair. We are interested in a wide variety of model systems and experimental approaches, including biochemistry, molecular biology, structural biology, animal models, genomics, proteomics, bioinformatics, and computational biology. The Green Center's research programs focus on, but are not limited to, reproduction and development in a broad sense, as well as aspects of endocrinology, stem cells, cancer, metabolism, inflammation, immunity, and neurobiology.

- ***Position 1*: Signaling, chromatin, and gene regulation** – a broad search for candidates using a wide array of experimental approaches to address fundamental questions in nuclear signaling, chromatin, transcription, epigenetics, and RNA biology.
- ***Position 2*: Genomic, bioinformatic, computational, and evolutionary approaches to understanding gene regulation** – a more focused search in areas that will connect to broader genomic initiatives on campus.
- ***Position 3*: Molecular biology of female reproductive systems** – a search for candidates using cell-based or physiological models in combination with molecular or genomic approaches to address fundamental questions concerning female reproductive biology.

Cecil H. and Ida Green Center for Reproductive Biology Sciences

UT SOUTHWESTERN
MEDICAL CENTER

The Green Center is an endowed basic science research center at UT Southwestern, which promotes and supports cutting-edge, integrative, and collaborative basic research in reproduction, development, and related areas of biology, as well as strong connections between basic and clinical research. This recruitment is part of a major university and department-supported renovation and rejuvenation of The Green Center over the past three years. Successful candidates will be housed in a newly renovated state-of-the-art research facility and provided a generous start-up package, and are expected to establish scientifically rigorous and externally funded research programs and participate in center, department, and university teaching and training programs. To learn more about The Green Center, visit: **http://www.utsouthwestern.edu/utsw/home/research/greencenter/**

Candidates must have a Ph.D. or M.D. or equivalent in a relevant field of study, postdoctoral or comparable experience, and a demonstrated record of research excellence. Applicants should send a letter of application, curriculum vitae, and a statement of planned research projects as pdf files to **GreenCenter@UTSouthwestern.edu**, indicating the position of interest (1, 2, or 3) in the subject line. Applicants should also arrange for three letters of reference to be sent directly to the above e-mail address. Review of applications will begin on October 15, 2013 and continue during the 2013 – 2014 academic year or until the positions are filled, although applicants are encouraged to submit their materials as soon as possible.

UT Southwestern is an Affirmative Action/Equal Opportunity Employer. Women, minorities, veterans, and individuals with disabilities are encouraged to apply.

NW240700R

HARVARD
Faculty of Arts and Sciences
CHEMISTRY & CHEMICAL BIOLOGY

Tenure-Track Assistant Professorship in Chemistry (Organic)

Harvard University

Candidates are invited to apply for a tenure-track assistant professorship in organic chemistry, broadly defined to include chemical biology, organic synthesis, organic materials, physical-organic chemistry, organometallic chemistry, and catalysis. The appointment is expected to begin on July 1, 2014. The tenure-track professor will be responsible for teaching at the undergraduate and graduate levels.

We are seeking candidates who have an outstanding research record and a strong commitment to undergraduate and graduate teaching. Doctorate required by expected start date.

Candidates should arrange to have three letters of recommendation sent independently and provide a curriculum vitae, statement of teaching philosophy, list of publications, and outline of their future research plans. All applications and supporting materials must be submitted via the ARIeS portal **(http://academicpositions.harvard/edu/postings/4914)** no later than **October 15, 2013.**

Harvard is an Equal Opportunity/Affirmative Action employer. Applications from women and minorities are strongly encouraged.

NW240022R

STOWERS INSTITUTE®
FOR MEDICAL RESEARCH

Faculty Position in the Life Sciences

The Stowers Institute for Medical Research invites applications for a faculty position in the Life Sciences. We anticipate making an appointment in 2014 at the rank of Assistant Investigator. Research programs of interest include, but are not limited to: computational biology, genomics, stem cell biology, regenerative biology, epigenetics, and cell biology. Our interests encompass a broad gamut of experimental organisms and approaches. The successful candidate will benefit from and complement the Institution's existing strengths in genetics and epigenetics, cell and chromosome biology, stem cells and regenerative biology, developmental biology and evolution, biochemistry and neuroscience.

The position is fully funded throughout the candidate's appointment. This includes $580,000 per year for full salary support and research funding, in addition to start-up funds and ongoing needs for equipment. The initial appointment is for 6 years and is then subject to renewal every 6 to 7 years. In total, the package for a junior position is more than $3.5 million over the first term and increases significantly after promotion. In addition, investigators may take advantage of exceptional Core facilities and technology centers staffed by over 100 scientists. Stowers investigators may also choose to teach or mentor pre-doctoral researchers from the Institute's Graduate School.

Candidates must have a Ph.D. or equivalent degree and postdoctoral experience demonstrating innovation and excellence in their field. Candidates will be expected to possess a long-term vision of their scientific interests, to establish a vigorous and innovative research program, and to actively contribute to the Institute's mission and collegiality.

Deadline for applications is November 1, 2013. Applicants should submit a CV, a research plan, and arrange for the submission of three letters of reference through our application page at: http://www.stowers.org/facsearch2014/home

Questions should be directed to the Search Committee Chair, Dr. Alejandro Sánchez Alvarado (facultysearch@stowers.org).

The Stowers Institute for Medical Research is proud to be an equal opportunity employer. All qualified applicants will be afforded equal opportunity regardless of race, creed, color, religion, gender, sexual orientation, pregnancy, national origin, age, disability, military status or any other status protected by law.

NW240653R

天津大學

Tianjin University

Tianjin University (TU) – School of Pharmaceutical Science and Technology (SPST) initiates an international faculty recruitment campaign with the goal of building a world-class school comprising global talent, a cosmopolitan environment and state-of-the-art infrastructure.

We seek junior and senior scholars with a demonstrated high level of excellence in research and dedication to teaching in English, to help build two major platforms: the **Institute of Drug Innovation and Development;** and, the **Institute for Molecular Design and Synthesis.**

Outstanding talent in all areas of pharmacy and molecular science are sought (e.g, pharmaceutics, pharmacology and toxicology, structural biochemistry, chemical biology, biochemical engineering, pharmaceutical process and formulation, pharma economics, regulatory science, public health policy, computational chemistry and biology, and material science).

SPST is a signal program of the new Tianjin campus development plan, housed in building 24 of the main Tianjin campus and outfitted for the highest international standard of modern research.

The program will be run in English for research, teaching and internal administrative activities. Student recruitment at the graduate (MS and PhD) level will be internationally competitive; the undergraduate program will provide top class instruction in English and a broad-based liberal education experience accessible to an international and Chinese student body.

Does this progressive and forward looking development interest you? Can you see yourself as a productive member/partner of the team that will realize this vision? Do you know an investigator who meets the high standards TU desires? Then contact us at **infospst@tju.edu.cn**

For full consideration, please submit before **September 22, 2013,** a current CV with personal references, a list of publications, a summary of research directions (2-3 pages), a statement of teaching interest (1 page) and up to 3 exemplary publications as a **single pdf file** *[LASTNAME_firstname_SPST_candidate.pdf]*

Competitive candidates will be invited to participate in SPST's Symposium on *Directions in Modern Pharmaceutical Science December 5-8 2013.*

TU-SPST is looking to find talent ready to change the landscape of higher education in China. Are you ready to be an element of change?

JP239677RL

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Faculty Position in Biophysics

University of Texas Southwestern Medical Center

The recently inaugurated Department of Biophysics at The University of Texas Southwestern Medical Center invites applications for tenure-track faculty positions at the level of assistant professor. We seek outstanding candidates who study molecular mechanisms of biological processes, *in vitro* or *in vivo,* using physical and quantitative approaches. We particularly encourage applications from those with backgrounds in single-molecule spectroscopy or quantitative imaging, but are interested in all excellent candidates regardless of technical approach. Scientists within the Department of Biophysics participate in a vibrant, interdisciplinary, interdepartmental, and highly collaborative research community within the University, and enjoy unparalleled access to state-of-the-art research cores in X-ray crystallography, macromolecular NMR spectroscopy, molecular biophysics, cellular imaging, protein chemistry and high-throughput screening.

Applicants should submit a *curriculum vitae,* a concise description of previous research accomplishments and future plans (3 page limit) and three letters of reference by **October 31, 2013** to http://academicjobsonline.org. The posting number is 3019. You may email questions to BiophysicsSearch@UTSouthwestern.edu. Attention: Michael Rosen, Chair, Biophysics Faculty Search Committee, U.T. Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-8816.

UT Southwestern Medical Center is an Affirmative Action/Equal Opportunity Employer. Women, minorities, veterans and individuals with disabilities are encouraged to apply.

NW240495R

Yale University - Chemical Biology Institute

The Chemical Biology Institute, a cornerstone of Yale's new West Campus research enterprise, invites applications for tenure track positions at the ASSISTANT PROFESSOR level to commence 1 July 2014.

Faculty associated with the Chemical Biology Institute will hold primary appointments in any of several life science or physical science departments within the Faculty of Arts and Sciences, the School of Engineering and Applied Science, or the Yale School of Medicine.

At this time, we seek to identify two (2) candidates whose research interests and accomplishments align best with the Institute and either the Department of CHEMISTRY (**www.chem.yale.edu/**) or the Department of MOLECULAR, CELLULAR, AND DEVELOPMENTAL BIOLOGY (**http://mcdb.yale.edu**).

Successful candidates will be creative teacher-scholars with reputations for outstanding research at the combined interface of chemistry, biology, and/or medicine, and must possess a Ph.D. in a relevant discipline. Applicants should create a profile at [**https://academicjobsonline.org/ajo/jobs/2993**] and upload a statement of research plans, curriculum vitae, and up to five reprints of published work(s).

Applicants should also arrange for three references to upload their letters of recommendation, and identify in their cover letter the department that best aligns with their research interests and training.

For further information, contact Paula Maher-Rivera at **paula.maher@yale.edu** or **PO Box 27392, West Haven, CT 06516-7392.** The review of applications will begin on **15 October 2013.**

Yale University is an affirmative action, equal opportunity employer. Yale values diversity among its faculty, students, and staff and strongly encourages applications from women and underrepresented minorities.

NW240609R

UNIVERSITY OF UTAH
SCHOOL OF MEDICINE

Senior Faculty Position in Chemical Biology

As part of a major, institution-wide effort to develop a world-class environment for chemical biology at the University of Utah, the Department of Biochemistry is searching for an established chemical biologist for a 12-month appointment as Associate or Full Professor in the Tenure-Track. We are especially interested in investigators who are using phenotypic screens to discover new biological pathways, targets, and lead compounds, although related areas of chemical biology will also be considered. Notably strong opportunities for synergy exist in the areas of cardiovascular, cancer, metabolic, and neurological disorders. The successful candidate will have demonstrated academic excellence in original research and be committed to education. The faculty position is associated with the Center for Chemical Biology and Drug Discovery, which is part of the Utah State Technology and Research (USTAR) initiative, a long-term, state-funded investment to strengthen Utah's knowledge economy and support the commercialization of novel technologies and therapeutics. The starting date is flexible. Applications will be reviewed as they are received. The position will remain open until filled. Contact: 801-585-2555.

Apply online at: **http://utah.peopleadmin.com/postings/26519**

The University of Utah is an Equal Opportunity/Affirmative Action employer and educator. Minorities, women, and persons with disabilities are strongly encouraged to apply. Veterans preference. Reasonable accommodations provided. For additional information: http://www.regulations.utah.edu/humanResources/5-106.html. The University of Utah values candidates who have experience working in settings with students from diverse backgrounds, and possess a strong commitment to improving access to higher education for historically underrepresented students. The University of Utah Health Sciences Center is a patient focused center distinguished by collaboration, excellence, leadership, and Respect. The University of Utah HSC values candidates who are committed to fostering and furthering the culture of compassion, collaboration, innovation, accountability, diversity, integrity, quality, and trust that is integral to the mission of the University of Utah Health Sciences Center.

NW240595R

Karolinska Institutet

seeks

Professor to Aging Research Center (ARC)

ARC is looking for a Professor in cognitive neuroscience with special focus on aging.

Read more about the job at
www.ki.se/job
Please submit your application no later than **october 22 2013.**

Karolinska Institutet

W240375R

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LOUISIANA STATE UNIVERSITY HEALTH SCIENCES CENTER

Shreveport

Vice Chancellor for Research

The LSU Health Sciences Center in Shreveport, LA is seeking candidates for the role of Vice Chancellor for Research. The individual selected for this position will provide executive leadership for research administration and planning, and will work with fellow leaders to strengthen the campus research enterprise and infrastructure for an innovative and multi-disciplinary research program across North Louisiana.

The Vice Chancellor for Research will lead the institution in all areas of research. The Vice Chancellor will represent the campus in matters related to research including federal and state agencies, other research institutions and the local community. A priority for the incumbent will be to facilitate translational research opportunities. Additionally, the Vice Chancellor will be the designated Institutional Official responsible for research across the organization.

The institution's 436 bed University Hospital serves an urban and rural population of approximately 2.5 million, encompassing 25,000 square miles in Louisiana, East Texas, and Southwestern Arkansas. The resources for basic and clinical research are excellent. We have a long history of successful research collaboration between the basic science and clinical departments. The Virginia Shehee Biomedical Research Institute is ~160,000 total square feet and includes wet lab and staffed core facilities, such as for cellular imaging, DNA array analysis, and mass spectroscopy-proteomics. For more information on this opportunity at LSUHSC please visit us at http://www.lsuhscshreveport.edu/VCRworkingProspectus .

Successful candidates must be an MD/PhD or MD with an outstanding record of scholarly achievement, including a history of independent federal research funding, serving as a principal investigator and having administrative experience relevant to clinical and basic research. Requirements include an understanding of the diverse forms of research and scholarship conducted at a comprehensive research university, and an informed perspective about federally sponsored programs, intellectual property, technology transfer and commercialization in the university setting.

The search is being led by Glenn Mills, MD, FACP, Professor of Medicine, Chief, Section of Hematology and Oncology and Director, Feist-Weiller Cancer Center. Interested candidates may submit curriculum vitae via email at ShvFacultyRecruiting@lsuhsc.edu . Materials that cannot be submitted electronically may be mailed to: Vice Chancellor for Research Recruitment, Human Resource Management, 1501 Kings Highway, Shreveport, LA 71103.

LSU Health Shreveport respects diversity in the workplace and is an Equal Opportunity Employer.

NW240605R

Memorial Sloan-Kettering
Cancer Center
The Best Cancer Care. Anywhere.

FACULTY POSITION
Structural Biology Program
Sloan-Kettering Institute

The Structural Biology Program of the Sloan-Kettering Institute (www.ski.edu) invites applications for a tenure-track faculty position at the Assistant Member level (equivalent to Assistant Professor). We are interested in individuals who have an outstanding record of research accomplishments. Areas of interest include X-ray crystallography, NMR spectroscopy, single-molecule imaging, as well as the interface of structural, chemical and computational biology. Faculty will be eligible to hold graduate school appointments in the Gerstner Sloan-Kettering Graduate School of Biomedical Sciences, the Weill Cornell Graduate School of Medical Sciences, as well as the Tri-Institutional MD/PhD Training Program.

The deadline for applications is **November 1, 2013**. Interested candidates should visit **http://facultysearch.ski.edu** to access the on-line faculty application. Please visit the site as soon as possible, as it contains important information on the required application materials, including deadlines for submission of letters of reference.

Informal inquiries may be sent to **Julie Kwan at kwanj@mskcc.org or Dr. Nikola Pavletich, Chair, Structural Biology Program at pavletin@mskcc.org.**

facultysearch.ski.edu

MSKCC is an equal opportunity and affirmative action employer committed to diversity and inclusion in all aspects of recruiting and employment. All qualified individuals are encouraged to apply and will receive consideration without regard to race, color, gender, gender identity or expression, sexual orientation, national origin, age, religion, creed, disability, veteran status or any other factor which cannot lawfully be used as a basis for an employment decision.

NW240383R

Massachusetts Institute of Technology

Come work with us!

Assistant Professors
Neuroscience and Human Cognition

The Department of Brian & Cognitive Sciences (BCS) (http://bcs.mit.edu) and **The McGovern Institute for Brain Research at MIT** (http://mcgovern.mit.edu) are looking to hire up to three tenure-track faculty at the assistant professor level who will work in one or more of the following four areas: i) *Neuroscience* in nonhuman animals, especially if the candidate's work bridges levels using a variety of tools and/or has relevance for understanding the neural basis of brain disorders. ii) *Computational* approaches to intelligence, cognition or neuroscience; an experimental component to the candidate's research would be viewed as a positive but is not necessary. An affiliation with Electrical Engineering and Computer Science, the Computer Science and Artificial Intelligence Laboratory (CSAIL), or other allied departments is possible. iii) *Human cognitive neuroscience*, including MRI, MEG, and neurophysiological approaches. iv) *Human cognition* using behavioral methods, especially in the areas of language and/or cognitive development.

Successful applicants are expected to develop and lead independent, internationally competitive research programs and to share in our commitment to excellence in undergraduate and graduate education by teaching courses and mentoring graduate and undergraduate research. PhD must be completed by start day of employment and some postdoctoral training is preferred.

Please submit application materials – cover letter, CV, statement of research and teaching interests and representative reprints – online at https://academicjobsonline.org/ajo/jobs/3081. Please state research area in cover letter. To help direct the application, applicants should indicate which of the four areas listed above is their main research area by answering the mandatory questions included in the application. In addition, please arrange to have three letters of recommendation submitted online. Review of applications will begin on **November 15, 2013.**

MIT is an affirmative action employer, and we encourage applications from women and underrepresented minorities.

http://web.mit.edu

NW240703R

The Department of Physiology (http://www.physio.northwestern.edu) of the Northwestern University Feinberg School of Medicine in downtown Chicago invites applications for a full time tenure track position at the Assistant - Full Professor rank in the area of cellular and/or systems neuroscience.

Applicants should have a Ph.D. or M.D. degree and an outstanding record of research in cellular and/or systems neuroscience that adds to existing departmental strengths in sensorimotor integration, pain perception, studies of neurodegeneration, and learning and memory. Individuals using animal models and innovative experimental techniques including biochemical or molecular approaches, optical imaging, optogenetics, multi-electrode recordings and behavioral measures are particularly encouraged to apply, but the scientific excellence of the candidate is more important than the particular area of research.

Applicants should send (as a single PDF) a cover letter, curriculum vitae, a two-page description of research accomplishments and plans, and the name and contact information for three references to **cellular-systems-neuroscience@northwestern.edu**. *Please quote reference number 21007.* Start date is negotiable.

Northwestern University is an Affirmative Action, Equal Opportunity Employer. Women and minorities are encouraged to apply. Hiring is contingent upon eligibility to work in the United States.

NW240654R

FACULTY APPOINTMENT IN CELL BIOLOGY
Department of Cell Biology
Duke University Medical Center

The Department of Cell Biology at Duke University Medical Center is hiring a tenure-track faculty member. The preferred level is for an Assistant Professor who would start his/her independent career at Duke.

Candidates with demonstrated success in addressing important and fundamental questions of developmental, regenerative, or cellular biology are encouraged to apply. Preference will be given to candidates whose activities complement and strengthen research programs within the department (**http://www.cellbio.duke.edu/**).

Successful candidates will have track records of creativity and productivity, demonstrated potential for outstanding future achievements, and a desire to participate in an exciting, interactive, and growing community. They will be expected to mount a productive and innovative research program, to obtain outside funding, and to participate actively in graduate training.

Candidates must have a Ph.D., M.D. or equivalent degree. Minority candidates are especially encouraged to apply. For full consideration, applications should be submitted by November 15, 2013, although applications will be considered until the position is filled.

Applicants should submit curriculum vitae, a brief summary of current and proposed research, and 3 letters of recommendation to **https://academicjobsonline.org/ajo/jobs/3082**

Questions may be directed to the Search Committee Chair, Ken Poss, Department of Cell Biology (**cellbiofacultysearch@dm.duke.edu**)

Duke University is an Equal Opportunity Affirmative Action Employer.

NW240746R

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STANFORD UNIVERSITY
Stanford Institute for Chemical Biology

The Stanford Institute for Chemical Biology is an independent institute at Stanford University, formed in partnership with the Schools of Medicine, Humanities and Sciences, and Engineering. The Institute is seeking applicants for a tenure-track faculty position at the junior level (Assistant or untenured Associate Professor). Candidates are expected to have earned a Ph.D. or M.D. degree in any discipline of science, engineering or medicine. We will consider candidates knowledgeable in the general area of chemical biology. All areas encompassed by the field of chemical biology are acceptable. In general, we give higher priority to the overall originality and promise of the candidate's work than to the sub-area of specialization.

The successful candidate will have his/her primary appointment in a department within the School of Medicine, Humanities and Sciences, or Engineering. He/she will be expected to teach and/or perform clinical service within this department in a manner that is consistent with standard practices for tenure-track faculty within that department. The candidate will also be expected to develop a world-class research program in chemical biology. Candidates should be seeking a stimulating interdisciplinary environment in which to pursue teaching and research. We anticipate that the faculty member will develop interactions with faculty not only in his/her home department but also in other departments and Schools at Stanford and at the Stanford Synchrotron Radiation Laboratory.

Applications should be addressed to Professor James K. Chen, Search Committee Chair and include a curriculum vitae, a description of future research plans, and a teaching statement. These materials should be submitted electronically as a single PDF to sicbsearch@stanford.edu by November 1, 2013 to ensure full consideration. Applicants should also have three reference letters submitted to sicbsearch@stanford.edu by the application deadline.

Stanford University is an equal opportunity employer and is committed to increasing the diversity of its faculty. It welcomes nominations of and applications from women and minority groups, as well as others who would bring additional dimensions to the university's research, teaching, and clinical missions.

NW240361R

College of
Biological Sciences
UNIVERSITY OF MINNESOTA
Driven to Discover℠

Are you a creative investigator who excels at working collaboratively and pushing disciplinary boundaries?

Then we want to hear from you.

The College of Biological Sciences at the University of Minnesota is hiring scientists for **three interdisciplinary research clusters in emerging areas of biology: genome variation, cellular biophysics and synthetic biology.**

All positions are tenure track. Successful candidates will have expertise that complements current faculty and be committed to graduate and undergraduate education.

Learn more at
z.umn.edu/cbsclusterhiring

The University of Minnesota is an equal opportunity educator and employer.

NW240431R

HARVARD
Faculty of Arts and Sciences
CHEMISTRY & CHEMICAL BIOLOGY

Tenure-Track Assistant Professorship in Chemistry (Inorganic)
Harvard University

Candidates are invited to apply for a tenure-track assistant professorship in inorganic chemistry, broadly defined to include catalysis, synthesis, mechanism, materials, and energy-related research. The appointment is expected to begin on July 1, 2014. The tenure-track professor will be responsible for teaching at the undergraduate and graduate levels.

We are seeking candidates who have an outstanding research record and a strong commitment to undergraduate and graduate teaching. Doctorate required by expected start date.

Candidates should arrange to have three letters of recommendation sent independently and provide a curriculum vitae, statement of teaching philosophy, list of publications, and outline of their future research plans. All applications and supporting materials must be submitted via the ARIeS portal **(http://academicpositions.harvard/edu/postings/4913)** no later than **October 15, 2013.**

Harvard is an Equal Opportunity/Affirmative Action employer. Applications from women and minorities are strongly encouraged.

NW240023R

BioTechMed®
GRAZ

BioTechMed-Graz is a cooperative initiative between the University of Graz, the Medical University of Graz and the Graz University of Technology at the interface of basic biomedical research, technological developments and medical applications.

With the cooperative project BioTechMed-Graz, the three partner universities are pursuing the goal of pooling their pre-existing competences in the four major research areas of 'Molecular Biomedicine', 'Neurosciences', 'Pharmaceutical and Medical Technologies' and 'Quantitative Biomedicine and Modelling'. To strengthen the cooperation, four junior research groups in the above-mentioned research areas will be implemented.

The following § 99 (1) UG professorships are to be filled:

Professorship of Neuroimaging –
Institute of Psychology at the University of Graz

Professorship of Interactive Microbiome –
Medical University of Graz

Professorship of Biopharmacy – Graz University of Technology

Professorship of Bioinformatics – Graz University of Technology

Necessary Qualifications:
- ➢ University degree in the subject area
- ➢ Scientific doctorate or relevant habilitation or equivalent qualification
- ➢ Pedagogical aptitude for university teaching
- ➢ Experience holding lectures in English is advantageous
- ➢ Competence in the field of gender mainstreaming
- ➢ International research activities
- ➢ Experience in the successful acquisition of third party funds
- ➢ Excellent publication record

40 hours/week; 5 year limited term employment contract according to § 99 (1) UG; expected to be filled starting February 1st 2014

Date for interviews and lectures of potential candidates:
21–23 November 2013

End of application deadline: **10 October 2013**

Link for further information: ***http://biotechmedgraz.at/de/***

W240688R

Contact Singapore
contactsingapore.sg

:insideview
profile feature

Pieter Eichhorn, *junior principal investigator at the Cancer Science Institute of Singapore, National University of Singapore.*

The son of a Dutch ship's captain, Pieter Eichhorn was born in Singapore, travelled through most of South East Asia as a child and eventually settled and grew up in Canada. After a PhD in the United Kingdom, he embarked on a research career at the Harvard Medical School, Massachusetts General Hospital and Vall d'Hebron Institute of Oncology. His research is focused on identifying the ways in which tumours become resistant to cancer therapies such as Herceptin. Here, Eichhorn explains why he decided to come back to Singapore in 2012 to set up his first lab as a principal investigator.

Q. What brought you back to Singapore?

What Singapore offered was, essentially, the best opportunity available to begin my career as a principal investigator (PI). I applied to about 20 different places in Europe and got a few job offers. The start-up packages they offered were pretty good but, essentially, a quarter of what Singapore was offering me—$300,000 per year for the first three years, salaries paid for by the university, all the basic lab equipment paid for by the institute. By comparison in the States, for example, a good start-up package would be about $500,000 in total. There are four major universities here - the National University of Singapore (NUS), the Nanyang Technological University of Singapore (NTU), Duke-NUS and Yale-NUS, and they all offer similar packages for a PI position.

Q. What is the research landscape in Singapore?

What Singapore has tried to do over the last 10–15 years is build a critical mass of scientists, so they initially invested a lot of money into that. Is the quality of science here comparable to the quality of science in Boston? I would say it's probably at about 70% of the quality. Singapore has done a fantastic job in recruitment—if there are any experts in the field that I need to question, everybody's readily available. I don't feel inhibited going to talk to them or setting up collaborations.

For example, the National University hospital has about 4,000 breast cancer tumour samples, which are made readily available to scientists working on relevant breast cancer research projects. We're looking at Herceptin resistance in the lab and those patient samples have been made available to me without any complications—the cancer physicians are very open to collaborating with the basic scientists.

Q. What are the funding opportunities for clinical scientists in Singapore?

I'm actually quite surprised by how much funding there is available. The Singapore government has put a huge amount of effort and funding into translational research, such as bench-to-bedside type grants and phase 1 trials.

Of the large grants that are available, maybe 20% of applications get funded here, compared with 7–8% in the US. Yes it is still competitive, but there's a lot more opportunity and funding available here than you would have in the US or the UK.

In Europe or the US, it's also harder to get funding as a young PI. Singapore offers a lot of lower-tiered grants—let's say $200,000 over two or three years—so there's a very good chance as a young PI to get a moderate amount of money into the lab, which is really what's required to get yourself established.

Q. What is the biggest downside of moving to Singapore?

The hardest part of coming from the US or Europe, especially if you have a family, is the distance. My father lives in Canada, it's a 24 hour flight, it's not easy to go see him.

Professionally, you might feel left out of the US/European hub in terms of getting invited to conferences and people from Europe trying to set up collaborations with you.

Q. Is it an expensive place to live?

The thing that is very expensive here is housing, so it's difficult to buy a place. As an assistant professor at the National University, the university has their own apartments and it's a bargain because the university subsidises more than half of my rental. That is for the first 9 years.

Buying a car here is virtually impossible, because they want to prevent traffic congestion on the roads. The cheapest car you can buy is about £60,000 [$91,000]. Public transport is fantastic though—the metro is one of the best I've seen, buses can take you anywhere and the taxis are incredibly cheap.

School fees can also be prohibitive. If you come here by yourself or with your partner, it's a fantastic place. You'll be living like kings. If you come here with children, and they go to international schools, it becomes very expensive. I have no problem in sending my kids to public schools here—from what I've seen they're fantastic—but they are primarily for Singapore citizens and permanent residents. If you are on an employment pass, you can apply through an annual centralised school admission programme where posting is based on the applicant's performance in an English and Mathematics test, intended residential area and available school vacancies.

Q. What advice would you give someone considering the move?

For a person in my position, as a young PI, I don't think there's a better opportunity available in terms of financial benefits. I'm able to do all the work I want to do and not worry about that side of it. And I have no complaints in terms of the science or job prospects available to me in the future.

I think before any young scientist moves to Singapore, they should look long and hard at the different offers that are on the table. Look at the guaranteed funding, what the package includes, what the potential is for collaboration, and importantly, for promotion. Contact Singapore is very good place to start to get a feeling of what is on offer and the lifestyle you can expect.

Start your search for career opportunities in Singapore:
www.contactsingapore.sg/jobs

Contact Singapore engages overseas Singaporeans and global talent to work, invest and live in Singapore.
For more information on working and living in Singapore, please visit **www.contactsingapore.sg**
You can also email our officers in London: **london@contactsingapore.sg**

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Clinical Science & Faculty Recruitment

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We welcome research talent and academics from Translational Researchers and Clinician Scientists to Post-doctoral Research Fellows, Principal Investigators and Professors (Assistant/Associate/Full) across all research fields.

Visit the *Singapore Pavilion* at Booths 34, 35 and 39 at the 2013 Naturejobs Career Expo to find out more about career opportunities in Singapore.

Contact Singapore together with our research and academic partners from Singapore will be on hand to answer any questions you have about working and living in Singapore.

For opportunities specific to clinical research, please visit
www.contactsingapore.sg/Clinical/UK2013

For opportunities in other research and academic areas, please visit
www.contactsingapore.sg/Research/Europe2013

Participating employers from Singapore:

Cancer Science Institute, National University of Singapore

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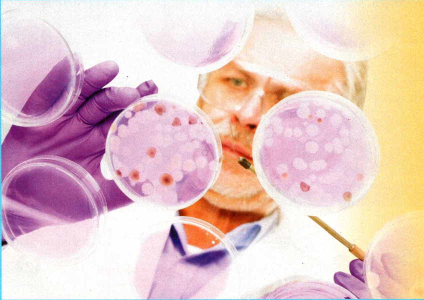
NUS Yong Loo Lin School of Medicine

Partners in Academic Medicine

SingHealth-Duke-NUS Academic Healthcare Cluster

Singapore University of Technology and Design



Not able to meet us in person?
Join us and our employer partners at the **Contact Singapore Virtual Career Fair** for Research and Academia in October 2013.

More details will be available on our website at
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For enquiries, email: **london@contactsingapore.sg**

Contact Singapore
contactsingapore.sg

U239499R

naturejobs
CAREER
EXPO
LONDON 2013

19 SEPTEMBER 2013

BUSINESS DESIGN CENTRE, LONDON

PREVIEW FEATURE

Dear *Naturejobs* reader,

This feature offers you a flavour of the 2013 *Naturejobs* Career Expo, brought to you by *Naturejobs,* the careers and recruitment section of Nature Publishing Group. The Expo and accompanying conference will take place on Thursday 19th September at the Business Design Centre in Islington, London.

The Expo is the UK's largest annual careers fair aimed at those in the life sciences, physical sciences, and medicine. The seventh in the series, this year is the largest one yet. What can you expect?

We have over 65 companies and organisations who will be taking over the exhibition hall ready to find out more about you and your skills. Our exhibitors are actively seeking scientists to fill a range of roles both in the UK and abroad, including Germany, Denmark, the US, Brazil and Russia. We have exhibitors from universities, the pharmaceutical industry, digital technology companies, science publishing and more.

During the course of the day, there will be eight workshops organised by some of the exhibitors to give you a chance to gain a more in-depth understanding of their aims, opportunities, and advice for your career development. Topics include how to get your research published in a top journal, managing your research career, and opportunities abroad.

As always, a highlight of the day will be the conference programme, running alongside the exhibition and workshops. The conference will cover a range of topics, presented by speakers whose careers span academia, industry, and the public and private sectors. Topics this year include how to transition from academia to industry, how to get a job in science communication, working in food and retail R&D, and entrepreneurship. Each of the sessions will allow plenty of time for questions, and we will also host an interactive room where delegates can continue the discussion with speakers following the sessions. We are delighted to welcome as our keynote speaker, to open the conference programme sessions, Eva Amsen, Outreach Director of *F1000Research.*

This year, conference delegates will also have the opportunity to take part in a one-on-one CV checking service, where careers experts will be on hand to share their advice to boost the potential of you CV. The service will be available on a first come first serve basis for conference ticket holders only.

We would like to thank all the individuals and organisations who have helped in developing the conference programme and format of the Expo and all our academic ambassadors. We also thank our Event Partners, our Gold and Silver sponsors, and all the other exhibitors for their support. The Expo is designed for you, the scientist, to explore, discuss, and seek out the opportunities that will take you on the next step of your career. Attending the Expo is free, and there is a small fee to attend the conference. To register, please visit **http://www.nature.com/natureconferences/njce2013/index.html.**

We look forward to seeing you on the day.

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Academics.com
www.academics.com/expo

ATASS Sports
www.atass-sports.co.uk

Bielefeld University
www.uni-bielefeld.de

Biosciences Careers Group
www.societyofbiology.org/education/careers/bioscience-careers-group

BIOSS Centre for Biological Signalling Studies
www.bioss.uni-freiburg.de

bizkaia:talent
www.bizkaiatalent.org

CEA-Enhanced Eurotalents
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careers.roche.com

Friedrich-Schiller University Jena
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German Academic Exchange Service (DAAD)
www.daad.org.uk

German Research Foundation (DFG)
www.dfg.de/en

German Scholars Organization (GSO)
www.gsonet.org

Goethe University Frankfurt
www2.uni-frankfurt.de/en?locale=en

Helmholtz Association
www.helmholtz.de

IK4 Research Alliance
www.ik4.es/en

Imperial College London - The Postdoc Development Centre
www3.imperial.ac.uk/staffdevelopment/postdocs1

International Graduate School in Molecular Medicine Ulm
www.uni-ulm.de/mm

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Keck Graduate Institute (KGI)
www.kgi.edu/academic-programs/postdoc-professional-masters-program-(ppm).html

Konstanz Research School Chemical Biology (KoRS-CB)
www.chembiol.uni-konstanz.de

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Singapore University of Technology and Design
www.sutd.edu.sg

SingHealth-Duke-NUS
www.singhealth.com.sg and www.duke-nus.edu.sg

Skolkovo Institute of Science and Technology
http://skoltech.ru

Switzerland
www.swissuniversity.ch

TalentScotland
www.talentscotland.com/lifesciences

Teach First
www.teachfirst.org.uk

Technical University of Denmark (DTU)
www.dtu.dk/English
www.dtu.dk/english/career

Technische Universität München (TUM)
www.tum.de

The Cancer Science Institute of Singapore
www.csi.nus.edu.sg

The European Molecular Biology Laboratory (EMBL)
www.embl.org

The Max Planck Society: Research for the Future
www.mpg.de/en www.imprs.brain.mpg.de

The Okinawa Institute of Science and Technology (OIST)
www.oist.jp

The São Paulo Research Foundation (FAPESP)
www.fapesp.br/en

Universität Heidelberg
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CONFERENCE TOPICS AND SPEAKERS

Conference Programme Overview
Catherine de Lange, *Naturejobs*

You're All Different: Creating Your Own Career
Eva Amsen, F1000 Research

Pharma
Nessa Carey, Pfizer, Inc

Academia "The Reality": Early Career
Julie Keeble, King's College London
Ryan O'Shaughnessy, University College London

Agri-Chemical Business
Hadyn Parry, Oxitec Limited

Academia "The Reality": Senior Roles
Amrita Ahluwalia, Queen Mary University of London
Sarah Gurr, University of Oxford
Kate Jones, University College London

CRO's
Elizabeth Allen, Quintiles Drug Research Unit at Guy's Hospital

Entrepreneurship
Andrew Mackintosh, Royal Society Enterprise Fund

Food/Retail R&D
Claire Hughes, Marks & Spencer.

Transferable Skills to Progress your Career
Anna Price, King's College London
Sarah Blackford, Society for Experimental Biology

SME'S "Size isn't Everything"
Philippa Ryan, Knowledge Transfer Partnerships

UKRSA – "By Researchers for Researchers"
Daniel Weekes, UKRSA

Academia to Industry
John Clarkson, Atlas Genetics Ltd

Science Communication
Jenny Gimpel, Great Ormond Street Hospital
Alok Jha, *The Guardian*
Marek Kukula, Royal Museums Greenwich
Chris Tyler, Parliamentary Office of Science & Technology

How to Write an Effective CV
Sarah Blackford, Society for Experimental Biology
Barbara Janssens, German Cancer Research Center (DKFZ Heidelberg)

NEW INTERACTIVE ROOM

The interactive room is a new addition to the Expo conference, which will help you make the most of your day.

Speakers and exhibitors will be available to answer your questions in small groups, giving you more time to get the information that's most important to you. This space will also serve as an After Talk area, where you can catch up with speakers after their session talks if any questions were left unanswered.

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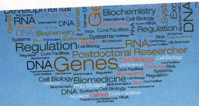

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To learn more, visit our booth # 21&22 at NJCE
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We will be at the Nature Careers Expo event and are always keen to speak to outstanding scientists with the talent and commitment to drive our research further. Why not come and see us to discuss our work and find out how you could be involved in the fight against cancer. Alternatively, find out more about our work and current opportunities at **www.paterson.man.ac.uk**

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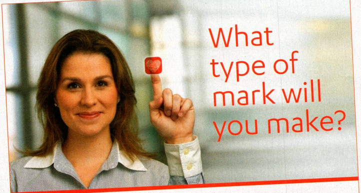
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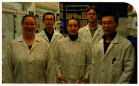

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Otsuka Pharmaceutical Co., Ltd and its group companies (http://www.otsuka.co.jp/en/) invite applications for the positions of scientist & clinical research associate to lead the global research and development of new drugs. Successful applicants will lead a team of passionate professionals who strive to advance science, technology, and medicine by developing products designed to help people live better lives.

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	Clinical Research Associates - Cancer
Experiments:	**Scientists -** PhD degree with two to five years combined post-doctoral and industrial experience, preferably in the pharmaceutical industry or biotechnology sector
	Clinical Research Associates - Extensive experiences of anti-cancer drug development and expertise of regulatory registration in Europe

Applicants should include curriculum vitae written in English, a list of publications, and a statement explaining former research/work experience.

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Applications should be sent to: **Email: recruitRD@otsuka.jp**

(All the applications should be converted to PDF)

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"The Naturejobs Career Expo is an important tool for VIB in getting more European visibility as an employer in life sciences. We were also impressed by the great organization and the friendly assistance of all "Nature" coworkers during the preparation phase and on the career day itself. That's why we will be coming back this year." **VIB**

"The Naturejobs Career Expo is a great platform to get to know qualified candidates from all over the world. We were pleasantly surprised by the internationality of the attendants! It allowed us to present the diverse research opportunities in Germany to a very multinational audience."
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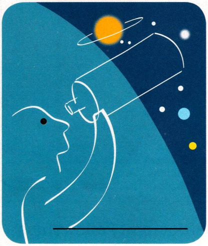

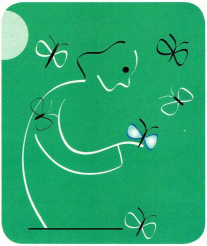

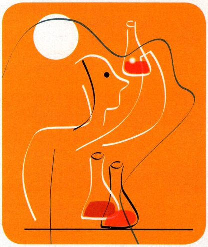

Fifty percent of all science created in Brazil is produced in the State of São Paulo. The state hosts three of the most important Latin American universities: Universidade de São Paulo (USP), Universidade Estadual de Campinas (UNICAMP) and Universidade Estadual Paulista (UNESP). Other universities and 19 research institutes are also located in São Paulo, among them the Technological Institute of Aeronautics (ITA), the National Institute for Space Research (INPE) and the National Synchrotron Light Laboratory (LNLS), besides most of Brazilian Industrial P&D.

The São Paulo Research Foundation (FAPESP), one of the leading Brazilian agencies dedicated to the support of research, has ongoing programs and support mechanisms to bring researchers from abroad to excellence centers in São Paulo.

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See more about it at **www.fapesp.br/yia**.

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For more information on ARRTI and additional positions/studentships, visit:
http://www. uleth.ca/artsci/arrti

The University of Lethbridge offers PhD studentships/assistantship opportunities in a range of multi-disciplinary areas in Sciences. Apply for a PhD program by September 30, 2013.

For more information or to apply contact the School of Graduate Studies:
www.uleth.ca/graduatestudies
Phone: 403 329-5194
Email: sgsinquires@uleth.ca

Consideration of applications will continue until the positions are filled between now and November 30, 2013.

NW234948R

Transforming Learning and Discovery

macmillan
Science and Education

MACMILLAN SCIENCE AND EDUCATION
SALES DIRECTOR, AUSTRALIA & NEW ZEALAND

SYDNEY, AUSTRALIA

Macmillan Science and Education, a global division of the family owned Holtzbrinck Publishing Group, is seeking a Sales Director to manage staff in Australia and New Zealand handling sales of textbooks and digital services in the higher education domain and online journals in science, social sciences, humanities and economics.

This is a challenging senior role that will require the successful applicant to oversee staff selling a broad portfolio of publications in higher education from Palgrave, Bedford, Freeman and Worth (Macmillan Higher Education), and a local Australian list; digital publications and services of Macmillan New Ventures, including *Nature Education, Sapling Learning, Late Nite Labs, PrepU*, *iclickers,* and *EBI Map Works; Palgrave Connect*, an ebook platform; and the online journals of Nature Publishing Group and Palgrave, including the world famous science journal *Nature*.

The Sales Director will be tasked to help bring about the digital transformation of Macmillan's higher education business in Australia and New Zealand and to build synergies and interactions between the campus team handling sales of textbooks and the online team selling journals and ebooks. This is a great opportunity for a highly motivated individual to assume a diversity of roles and to inspire sales staff to take on new challenges.

This position will be based in Sydney reporting to the Managing Director of Macmillan Science and Education, Australia and New Zealand, who is also based in Sydney, and will require management of staff in Sydney, Melbourne, Brisbane and Auckland.

To apply, please submit your CV and a cover letter via our online recruitment system here: **http://jobs.macmillan.com** Please quote ref: MSE/001/13 and provide an indication of your current salary and salary expectations for this position.

Closing date: 20th September 2013

IN240593R

WWW.NIH.GOV
Positions@NIH

THE NATIONAL INSTITUTES OF HEALTH

The NIH Intramural Research Program is Recruiting Tenure-Track "Earl Stadtman Investigators"

The National Institutes of Health, the U.S. government's premier biomedical and behavioral research enterprise, is pleased to announce its fifth annual call for "NIH Earl Stadtman Investigators," a broad recruitment of tenure-track investigators for all NIH intramural programs. Scientific discoveries from our intramural laboratories, with their extensive infrastructure and critical mass of expertise, have a crucial role in both maintaining America's research excellence and advancing medical treatments and cures.

Come join the team whose hallmarks are stable funding, intellectual freedom, shared resources, and access to a wide range of scientific expertise. We seek creative, independent thinkers eager to take on high-risk, high-impact research. A fantastic array of early-career scientists already have been hired through the "Stadtman" recruitment in the last four years.

A variety of basic and translational/clinical positions are available, with areas of active recruitment including (but not limited to): Biochemistry, Biomedical Engineering, Biophysics, Biostatistics, Cancer Biology, Cell Biology, Cell Metabolism, Chemical Biology, Chromosome Biology, Circadian Biology, Computational Biology, Developmental Biology, Epidemiology, Genetics, Genomics, Health Disparities, Hearing & Balance, Immunology, Infectious Diseases, Microbiology, Molecular Pharmacology, Neurodevelopment, Neurosciences, Sensory Biology, Structural Biology, Systems Biology, Toxicology, Translational Research, and Virology.

*****

Who we are: Among our approximately 1,100 principal investigators and 5,000 trainees are world-renowned experts in basic, translational, and clinical research. Our strength is our diversity in pursuit of a common goal, to alleviate human suffering from disease. Similar to academia, we offer our scientists the opportunity to mentor outstanding trainees at all levels (e.g., graduate students and postdoctoral fellows) in a research setting.

Whom we seek: For this broad, trans-NIH recruitment effort, we seek talented, early-career scientists with a clear and creative research vision who wish to contribute to the nation's health.

Qualifications/eligibility: Candidates must have an M.D., Ph.D., D.D.S./D.M.D., D.V.M, D.O., R.N./Ph.D., or equivalent doctoral degree and have an outstanding record of research accomplishments as evidenced by publications in major peer-reviewed journals. Applicants may be in early stages of their research careers or non-tenured early-to-mid career scientists. Appointees may be U.S. citizens, resident aliens, or non-resident aliens with, or eligible to obtain, a valid employment-authorization visa.

How to apply: Applicants must submit four items: (1) a CV; (2) a three-page proposal titled Research Goals, i.e., the research you hope to perform at the NIH; (3) a one-page statement titled Long-term Research Vision and Impact, i.e., what you hope to achieve for yourself, your field, and society; and (4) contact information for three professional references. Submit these through our online application system at http://tenuretrack.nih.gov/apply between August 1 and 11:59 p.m. EDT October 1, 2013. You will be asked to designate a primary and secondary scientific area of expertise to aid in assigning your application to the appropriate review committee. Requests for letters of recommendation will be sent to your references when you submit your application. Reference letters will be accepted via upload to the website until 11:59 p.m. EDT October 15, 2013. We cannot accept paper applications.

What to expect: Search committees of subject-matter experts will review and evaluate applicants based on publication record, scientific vision and potential scientific impact of current and proposed research, demonstrated independence, awards, and references. The committees will identify the most highly qualified candidates to invite to the NIH for a lecture in November or December 2013, open to the NIH scientific staff, and for subsequent interviews with the search committees. Search committee chairs and NIH Scientific Directors, who lead our intramural programs, will identify finalists for possible recruitment as Earl Stadtman Investigators. Candidates not selected as finalists can be considered for other open NIH research positions. The entire process from application review to job offer may take several months, depending on the volume of applications.

*****

We call upon individuals who will open our eyes to possibilities we haven't yet envisioned, to complement our scientific mission and enhance our research efforts. More information about our program is at http://irp.nih.gov. The inspiring story of Earl and Thressa Stadtman's research at the NIH is at http://history.nih.gov/exhibits/stadtman. Specific questions regarding this recruitment effort may be directed to Dr. Roland Owens, Assistant Director, NIH Office of Intramural Research, at owensrol@mail.nih.gov. DHHS and NIH are Equal Opportunity Employers.

THE NIH IS DEDICATED TO BUILDING A DIVERSE COMMUNITY IN ITS TRAINING AND EMPLOYMENT PROGRAMS

NW238814R

KOREA UNIVERSITY Faculty Openings

Korea University, in Seoul, South Korea, seeks candidates to join the faculty in the spring semester of 2014 beginning March 1. We are recruiting enthusiastic, well-trained scholars who wish to refine their teaching experience and research in this part of the world. Korea University, founded in 1905, is ranked one of the top universities in Korea by major global assessment reports. The positions available for the candidates are assistant, associate, or full professors (a two or three year tenure track appointment, OR non-tenure track appointment). All candidates are expected to have ample university-level teaching experience, a strong commitment to excellence in scholarship, and dedication to teaching and research in their fields.

[Online Application]

- Applicants are asked to submit their applications electronically by using the website at http://kuweb.korea.ac.kr/faculty
- Application Deadline : Otober 16, 2013

[Point of Contact]

- Phone : + 82 - 2 - 3290 - 1072
- Fax : + 82 - 2 - 929 - 9164
- E - mail : faculty@korea.ac.kr

KOREA UNIVERSITY

KOREA
UNIVERSITY

JP240577R

CANCER RESEARCH UK | BEATSON INSTITUTE

The Beatson Institute for Cancer Research, Glasgow, UK

Drug Discovery Programme - Head of Biology

The Beatson Drug Discovery Programme (DDP) has been established as an Industry Standard Unit at the forefront of cancer drug discovery. Exploiting the basic biology strengths within the Beatson Institute and wider Cancer Research UK network, our portfolio includes some of the most exciting and challenging cancer targets.

We are seeking a highly motivated and talented scientist for the post of Head of Biology. Ideally you will have extensive industrial experience in small molecule drug discovery, having directed programmes from early stage Target and Hit Identification through to Lead Optimisation and possibly beyond. You will work closely with academic Group Leaders to identify new targets for the Beatson DDP as well as directing the screening, assay development and in vitro and in vivo aspects of the programme. Reporting directly to the Head of Drug Discovery, and together with the Head of Medicinal Chemistry and Structural Biology, you will have a key role in directing the overall strategy and success of the programme.

A Ph.D. in a relevant discipline plus extensive experience of small molecule drug discovery in an industrial environment is essential together with strong leadership, management and interpersonal skills.

In return you will receive a competitive salary commensurate with your skills and experience and find the Beatson an inspiring, supportive and creative atmosphere where your contribution will really make a difference.

Applications with CV and names of three referees should be sent to beatson@beatson.gla.ac.uk Informal enquiries are welcome and should be addressed to Dr Martin Drysdale, Head of Drug Discovery, m.drysdale@beatson.gla.ac.uk

Closing date for applications: 18th October 2013

Scottish Charity No. SC006106

U240675R

INSTITUTE for PROTEIN RESEARCH
OSAKA UNIVERSITY

Call for Applications for International Collaborative Research at Institute for Protein Research (IPR), Osaka University

The research should be conducted in the form of a collaboration including at least one of the Principal Investigators (PIs) at IPR, or it should use particular experimental facilities of IPR, including solution- and solid-state-NMR spectrometers with ultra-high magnetic fields, and a synchrotron beam line for biological macromolecular assemblies at SPring-8. The research period is **from April 1, 2014 to March 31, 2015.** IPR will support each project for visiting IPR. Each applicant should submit an application form for the collaborative research along with a recommendation letter from the PI of IPR. **The dead-line for submission is December 2, 2013.**

The application form and the precise information are available at our web page: **http://www.protein.osaka-u.ac.jp/eng/applications/applications/**
Contact person: Yuji Goto, Prof., IPR, Osaka University,
3-2 Yamadaoka, Suita, Osaka 565-0871, Japan.
FAX: +81-6-6879-8616, **E-MAIL:** yogto@protein.osaka-u.ac.jp

JP240574R

www.naturejobs.com

GRADUATE SCHOOL OF Quantitative Biosciences Munich

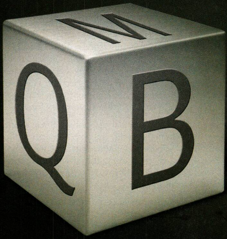
LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Do you wish to ...

... earn a PhD, building on your training in biochemistry, biology, physics, or applied maths?

... conduct cutting-edge research at the interface of experiment and quantitative theory?

... learn how to communicate and work with scientists from different fields?



Then join us at QBM!

The newly established Graduate School of Quantitative Biosciences Munich (QBM) is funded by the German Excellence Initiative and seeks to prepare young life scientists for the emerging era of quantitative, systems-oriented bioscience. It provides an innovative, international PhD training program that bridges the divide between traditionally separate disciplines, from biochemistry and medicine to bioinformatics, experimental and theoretical biophysics, and applied mathematics.

Key elements of the program are an interdisciplinary research project jointly supervised by two PIs from different fields, and an educational curriculum centered around an intensive core course that integrates a wide range of approaches to biological problems. A multi-facetted mentoring and professional skills program supports the students' growth as independent scientists.

For more information, visit us at **www.qbm.lmu.de**
Application deadline: **January 6, 2014**

W240689R

MACMILLAN
SCIENCE COMMUNICATION

Consultant Editor, Macmillan Science Communication | London, Shanghai, Tokyo

Can you help scientists maximise the impact and influence of their research? Macmillan Science Communication (MSC), part of the Nature Publishing Group (NPG) family, has a unique opportunity for an outstanding scientific journal editor to deliver and develop our training and editing services for researchers. Channelling the broad expertise within NPG, the Consultant Editor will promote best practices in scientific publishing to inquisitive researchers across the globe.

What you will be doing

- In conjunction with current NPG editors, leading the delivery of face-to-face training workshops worldwide
- Working with the Product Manager to develop existing, and create new, training materials for researchers with a range of backgrounds and abilities
- Overseeing the day-to-day management of MSC's Scientific Editing service, as applied to journal manuscripts, grant applications and other scientific texts
- Editing scientific text in a broad range of fields and consulting directly with authors
- Evaluating the effectiveness of and continuously improving MSC's services in response to client needs and user feedback
- Working with the Publishers to uncover new opportunities and to grow MSC/NPG revenues and reputation globally

What we would like you to have

- A PhD in a scientific discipline (required), and postdoctoral research experience and a record of publication (preferable)
- Experience of editorial decision-making at a high-impact scientific journal, and of developmental editing (e.g., primary manuscripts or reviews)
- Demonstrable experience of presenting or teaching in a lecture/seminar-style environment and an appreciation of the diversity of adult learning needs
- An advanced awareness of the research ecosystem (including the scholarly publishing landscape), and researchers' priorities, challenges and unmet needs
- Excellent interpersonal, organisational and communication skills

The role will suit you if

- You have a passion for communicating science both verbally and in writing
- You have an affinity for listening to, advising and sharing your knowledge with others
- You have a willingness to travel internationally, representing the organisation in an ambassadorial role
- You have a naturally gregarious disposition and enjoy being in front of an audience

This role is offered on a full-time, permanent basis and could be located in our London, Shanghai or Tokyo offices. The salary and benefits will be competitive.

Who we are

Macmillan Science Communication (MSC) and Nature Publishing Group (NPG) are part of Macmillan Science and Education. Macmillan Science and Education pushes the frontiers of learning and discovery by providing the best content and most innovative technology solutions to students, teachers and researchers. We place their needs at the centre of all we do and share their passion for transforming lives around the world.

Closing date: 25th September 2013

Application is by way of CV and covering letter quoting reference number **NPG/148/13** in the subject header via http://jobs.macmillan.com.

nature publishing group npg

IN240668R

Centre for Genomic Regulation Head of Bioinformatics

CRG Centre de Regulació Genòmica

Head of Bioinformatics Unit at CRG, Barcelona ref. HBU 0913

The Centre for Genomic Regulation (CRG, www.crg.eu), located at the Parc de Recerca Biomèdica de Barcelona (PRBB, www.prbb.org), is a leading Institute in biological and biomedical research. The CRG includes 4 research Programmes: Gene Regulation, Stem Cells and Cancer, Cell and Development Biology, Systems Biology, and Bioinformatics and Genomics, and has a partnership with the EMBL through the Systems Biology Programme. The PRBB incorporates as well the department of Life Sciences of the University Pompeu Fabra (CEXS/UPF, www.upf.edu/cexs), the Municipal Institute of Medical Research (IMIM, www.imim.es), and the Centre for Regenerative Medicine (CMRB, www.cmrbarcelona.org), in an international and collaborative scientific environment. Research at the CRG is supported by state-of-the-art core facilities including genomics (next-generation sequencing and microarrays), proteomics, bioinformatics, advanced light microscopy, biomolecular screening and protein technologies, and FACS. The CRG is looking for candidates to fill the following vacancy:

The **Bioinformatics Core Facility** (www.crg.eu/bioinformatics_unit) is dedicated to providing support and expertise in programming and advanced data analysis to the PRBB as well as to other research institutes. Services provided include consultation on bioinformatics methods and software, development and implementation of pipelines for the analysis of genomics, transcriptomics, and proteomics datasets, as well as data management and customized downstream data interpretation, software and database development, and the provision of access to computing resources, databases and specialized software. A major task of the Bioinformatics Core Facility is to train biologists in the use and application of bioinformatics tools for their research. The Bioinformatics Core Facility comprises the Unit Head and 3 experienced bioinformaticians who collaborate closely with the other Core Facilities and various CRG research groups. There is strong interaction regarding data sharing and software development with the groups of the Bioinformatics and Genomics program (http://big.crg.cat).

The mission of the **Head of the Bioinformatics Core** will be to scientifically and administratively manage the core facility. Daily operational tasks include discussion with scientists, project planning and budget management. Additionally, the Unit Head is expected to organize a training curriculum, and to offer tutorials and courses to the scientific community. It is the Unit Head's responsibility to ensure that the services offered by the Unit are kept state-of-the-art, to ensure efficient use of resources, and to define a strategy for new application developments based on the scientific orientation and future requirements of the CRG/PRBB. Establishing excellent relations with researchers, the CRG IT group and the other administration departments, as well as with software companies will be essential.

We are looking for a scientist with long-standing knowledge and experience in bioinformatics, with a Biology or Bioinformatics background. The candidate should have project management skills and be service-oriented and motivated to take on the challenge of leading a core facility and providing services to a large, international scientific community. Knowledge of Database Programming, Genome assembly and annotation, NGS and Proteomics analyses, Perl or Python, R programming and UNIX are expected. Communication skills, an open personality, and the capability to easily interact with people of different scientific backgrounds, nationalities and personalities, are fundamental requirements for this post. We offer a competitive salary with an open-ended contract, subjected to periodical external evaluations. Candidates should send their application, including their CV, a description of the proposed plan for the organization of the Unit, and the contact details of at least 3 potential referees to: **rrhh@crg.es** indicating the reference in the subject line.

Applications Deadline: **15th of October**

W240720R

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nature COMMUNICATIONS

Assistant, Associate or Senior Editor (Biological Sciences)

Nature Publishing Group (NPG) is a division of Macmillan Publishers Ltd, dedicated to serving the academic, professional scientific and medical communities.

Nature Communications is an innovative multidisciplinary science journal, which launched in April 2010. The journal publishes high-quality research from the breadth of the natural sciences and has already developed a strong track record in these disciplines. The journal offers rapid publication in an online environment, with Open Access options.

We now have an exciting opportunity for a biology editor to join our editorial team. The ideal candidate will have a PhD (or equivalent), and preferably postdoctoral experience, in an area of the biological sciences. Editorial experience would be beneficial, but is not required.

Reporting to the Managing Editor (biological sciences), the successful candidates will play an important role in determining the representation of their field in the journal and will work closely with the other editors on all aspects of the editorial process, including manuscript selection. This role will also entail significant liaison with the scientific community, the Nature research journal Chief Editors (UK and US) and the Editorial Advisory Panel.

This is a demanding and intellectually stimulating position. Broad scientific knowledge and training, excellent literary skills and a keen interest in the practice and communication of science are a prerequisite. The successful candidates must therefore have excellent communication and interpersonal skills. The salary and benefits will be competitive, reflecting the critical importance and responsibilities of this role.

This role is offered on a full-time, permanent basis and is located in our London office.

All applicants must be able to demonstrate the right to live and work in the UK in order to be considered for the position in the UK.

Closing date: 29th September 2013

Applicants should include a CV, a covering letter explaining their interest in the post and a Research Highlights piece in the style of *Nature Cell Biology* (300 words) on a recent paper from a related discipline to http://jobs.macmillan.com. Please quote reference number **NPG/149/13** in the subject header.

nature publishing group npg

IN240692R

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MRC | Laboratory of Molecular Biology

Cambridge, UK

Programme Leaders in Neurobiology

The Division of Neurobiology of the Medical Research Council Laboratory of Molecular Biology (MRC-LMB) wishes to recruit outstanding scientists who will develop an independent programme of research in the area of neurodegenerative diseases, with an emphasis on molecular/cellular mechanisms. Synergy with existing research programmes would be an advantage, but scientific excellence and the potential for major impact are paramount. Current research in the Division includes protein misfolding in relation to neurodegenerative diseases, mechanisms of synaptic transmission and vesicle recycling, as well as neural circuits, including sensory and motor processing, and circadian rhythms (www2.mrc-lmb.cam.ac.uk/NB/).

The LMB provides an excellent environment for hands-on research, with a new building providing outstanding facilities. The MRC has made a major investment in Neurobiology at the LMB, supporting the expansion of research in areas including neural circuits and neurodegenerative diseases, in addition to existing programmes. The MRC provides long-term support for ambitious and demanding projects, while administrative duties are minimal and no teaching is required. Interactions across the four Divisions of the LMB are encouraged. There is extensive central support, including electronic and instrumentation workshops, imaging and transgenic mouse facilities. The University of Cambridge and affiliated institutions form a vibrant Neuroscience community (http://www.neuroscience.cam.ac.uk/).

Candidates should have a PhD and/or MD and will have completed a period of postdoctoral training or equivalent, with an excellent track record, and show outstanding potential for independent research. The successful candidate will lead a small team and substantial funding will be available.

These appointments will be made at either Programme Leader-track or Programme Leader level, depending on achievements and experience. Programme Leader-track appointments will be made for those who demonstrate the potential to develop into Programme Leaders within six years. Candidates for Programme Leader positions should have a strong international track record of relevant independent research and a proven ability to lead a research team, pursuing original approaches to long-term research goals. Salaries are competitive (Programme Leader-track £35,935-£48,000 per annum; Programme Leader £47,699-£70,000 per annum).

These positions will remain open until they are filled; applications received by 15th October 2013 will be given priority.

Informal enquiries can be addressed to Michel Goedert (mg@mrc-lmb.cam.ac.uk).
For further information about the MRC visit www.mrc.ac.uk

Applications should include a covering letter and full CV, an outline of current research interests (1 page) and a proposal for future research (up to 2 pages), along with the names and addresses of three professional referees who have agreed to be contacted prior to interview.

Applications are handled by the UK Shared Business Services Ltd; to apply please visit our job board at http://www.topcareer.jobs/Vacancy/irc107173_3525.aspx.
If you are unable to apply online please contact 01793 867003 quoting reference IRC107173.

This position is subject to pre-employment screening
The Medical Research is an Equal Opportunities Employer No agencies please

U240677R

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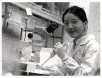

Faculty Positions at School of Chemical and Biomedical Engineering (SCBE)

Nanyang Technological University (NTU) in Singapore is ranked 47th in the QS World University Rankings 2012. SCBE at NTU invites applications for Assistant, Associate or Full Professors. For more information, visit www.scbe.ntu.edu.sg/About_Us/Pages/Open_Positions.aspx

Research Areas

Chemical and Biomolecular Engineering Division
- Process systems engineering
- Product and process design
- Pharmaceutical engineering
- Food engineering
- Separation technology
- Colloid and interface science
- Synthetic Biology

Bioengineering Division
- Therapeutic and diagnostic devices
- Biomechanics
- Bio-imaging and biosignal processing
- Biofluidics
- Neuro bioengineering
- Nature inspired bioengineering
- Mechanobiology of cell-cell and cell-matrix interactions

Application Details
Guidelines: www.ntu.edu.sg/ohr/Career/SubmitApplications/Pages/Faculty.aspx
Email: scbe_recruit@ntu.edu.sg

www.ntu.edu.sg

JP240548R

DUKE NUS
GRADUATE MEDICAL SCHOOL SINGAPORE

Assistant/Associate Professor Bioinformatics

The Duke-NUS Graduate Medical School, Singapore (Duke-NUS) seeks highly qualified candidates in bioinformatics and computational biology for a tenure-track assistant or associate professorship in its **Centre for Computational Biology** (see **http://www.duke-nus.edu.sg/research/centers/centre-computational-biology**). Candidates should have a Ph.D. or the equivalent, a strong publication record, and the desire to balance collaborative research with an independent research program. We seek candidates who focus on bioinformatics applied to human disease at a basic, translational, or clinical level.

We offer full salary support and a generous startup package including 5 years of research support, and candidates will be expected to establish an internationally competitive, independent research program. There are several sources of extramural funding within Singapore and our faculty has had good success in applications for these funds. In addition, the candidate will have opportunities to collaborate with faculty in Duke-NUS's five signature research programs: Cancer & Stem Cell Biology, Cardiovascular & Metabolic Disorders, Emerging Infectious Diseases, Health Services & Systems Research, and Neuroscience & Behavioral Disorders.

Duke-NUS is unique in bringing post-baccalaureate, research-intensive medical education to Asia. We represent a global partnership between two leading universities: National University of Singapore and Duke University, North Carolina, USA. Duke-NUS shares a modern campus with Singapore's largest hospital and several national medical research centers. We have strong research ties to SingHealth, one of Singapore's major healthcare providers, to other major research institutions in Singapore, and to Duke University. Duke-NUS offers both MD and PhD degree programs (see **www.duke-nus.edu.sg**).

Singapore is a modern, cosmopolitan, multicultural city and country with a population of 5.5 million and a per capita GDP higher than that of the US. English is an official language and spoken almost universally.

How to apply: Interested candidates should send a full application with CV, a statement of research interests, and the names of three referees to the Centre for Computational Biology, Duke-NUS Graduate Medical School Singapore via email to:
faculty.ccb@duke-nus.edu.sg

Applications will be accepted until the position is filled, with review to begin Dec 1, 2013.

Transforming Medicine, Improving Lives

Partner in Academic Medicine: SingHealth

JP240360R

Newcastle University

Faculty Fellowship Programme

£27,854 - £29,541, with progression to £33,230

Newcastle Biomedicine is seeking highly talented and motivated postdoctoral scientists who plan to apply to national fellowship schemes at all levels. We offer salaried positions that provide up to four years underpinning support to enable external fellowship applications to be made. You will have a strong publication record and be highly competitive for external fellowship funding. You will be mentored by experts and work with our leading researchers to develop independently led projects taking full advantage of the scientific and translational culture in the Faculty of Medical Sciences.

This opportunity is available through our ongoing Fellowship Programme which aims to support and develop future research leaders. By the end of the Newcastle University fellowship, the successful programme participant will have secured external funding and be supported through to the next stage of their career.

Newcastle University has established an excellent reputation, spanning fundamental life sciences, and clinical and allied healthcare sciences.

The Programme is supported by the Barbour Foundation and the Wellcome Trust's Institutional Strategic Support Fund.

Further details can be obtained from Professor Deborah Henderson, Associate Dean of Research, email: deborah.henderson@ncl.ac.uk

Please apply on line at http://www.ncl.ac.uk/vacancies/
Closing date: 29 September 2013.
Interview date: w/c 14 October 2013.
Job reference: A1616R (FMS).

U240719R

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BIGELOW LABORATORY FOR OCEAN SCIENCES

Two Principal Investigator Positions, Bigelow Laboratory for Ocean Sciences

Bigelow Laboratory invites applications for two Senior Research Scientist positions associated within the three centers at the Laboratory's new research campus: 1) Blue Biotechnology, 2) Ocean Biogeochemistry and Climate Change, and 3) Ocean Health. Bigelow research generally focuses on marine microbial processes, covering a broad range of topics as well as cross-cutting themes, all listed at the below web site.

Minimum requirements are a Ph.D. with two years of postdoctoral experience. Bigelow Laboratory is a primarily soft money institution with the expectation that the PI will generate external funding and be an active participant in the Laboratory's teaching and technology transfer activities. Salary and start-up packages are negotiable. The Laboratory provides generous medical/retirement benefits.

For complete information and application details, see: http://www.bigelow.org/about-bigelow-laboratory/position_openings/2pi_positions/ referring to Job # SRS-2013-1. For full consideration, the application should be received by 25 October 2013. The search will continue until the positions are filled, with a potential start date of summer 2014.

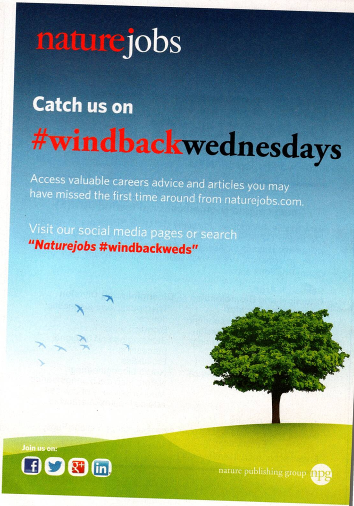
Bigelow Laboratory is an
Equal Opportunity/Affirmative Action institution.

NW240473R

"When trying to recruit good scientists for such a remote institutions like ours, in the Canary Is., the high visibility and wide-range exposure that Naturejobs provides has been crucial. I'm very happy to have international candidates who applied to our position from places ranging between St. Petersburg and Cincinnati. Thanks a lot!"

Eduardo Salido, MD, PhD,
Hospital Universitario de Canarias

naturejobs



naturejobs

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#windbackwednesdays

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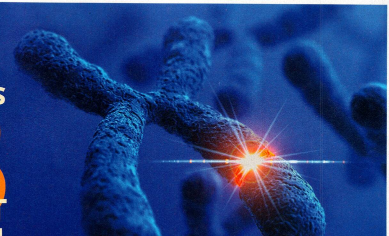
"Naturejobs #windbackweds"



CONGRATULATIONS 2013 WARREN ALPERT FOUNDATION PRIZE RECIPIENTS

DAVID BOTSTEIN, PHD
Princeton University

RONALD W. DAVIS, PHD
Stanford University School of Medicine

DAVID S. HOGNESS, PHD
Stanford University School of Medicine

Honoring their seminal contributions to the concepts and methods of creating a genetic map in the human, and of positional cloning, leading to the identification of thousands of human disease genes and ushering in the era of human genetics.

Call for 2014 Prize Nominations

The Warren Alpert Foundation's Scientific Advisory Committee, comprising internationally renowned biomedical scientists and chaired by Dean Jeffrey S. Flyer, requests nominations for the 2014 Warren Alpert Foundation Prize. Nominations should be made for scientific achievements that have led to the prevention, cure or treatment of human disorders or for seminal scientific findings that hold great promise of ultimately changing our ability to treat disease. Nomination Deadline: November 1, 2013

For more information, visit www.warrenalpert.org

WARREN ALPERT FOUNDATION

HARVARD MEDICAL SCHOOL

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Nominations for Young European Astronomers

FONDATION MERAC is a non-profit foundation started in 2012 with headquarters in Switzerland to recognize and support young European astronomers. There are yearly three MERAC Prizes awarded by the European Astronomical Society (EAS). The prizes of €20'000.– are for each of the three categories: Theoretical Astrophysics, Observational Astrophysics, and New Technologies (Instrumental/Computational).

The EAS Council invites EAS members to nominate suitable candidates for the MERAC Prizes of 2014. This being an even year, the three prizes will be **Best Doctoral Thesis Prizes** for degrees awarded during the period June 2010 – July 2013.

Monday, September 30, 2013 at 16:00 CEST is the strict deadline for nominations at: http://eas.unige.ch/merac_prizes.jsp

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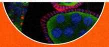

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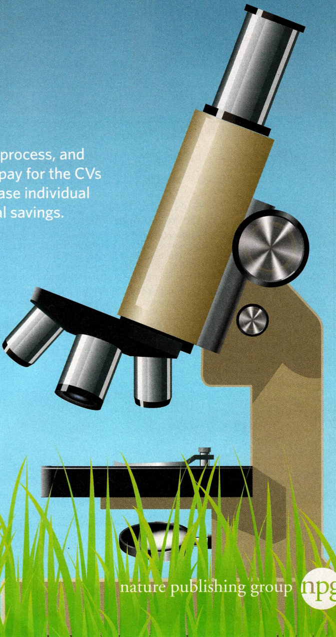

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LACUNAE

The rhythm of life.

BY WILLIAM MEIKLE

What Frank Green did wasn't difficult. All it took was a large amount of computing power and many weeks of meticulous research in musty book stacks. By mid-July he felt ready to test his findings.

He wasn't sure quite what he'd found, but the pattern was unmistakable. For most of recorded history, people have been disappearing without trace, often right in front of astonished friends and acquaintances.

The pair of professors tasked with reviewing Frank's work proved sceptical in the extreme, even after he showed them the simulation — the one that tracked the lacunae over time and space, the one that then predicted where and when these holes in space and time might occur. But two days later Frank was in Alberta, standing outside his tent where he'd bivouacked halfway up a deer trail at nearly 6,000 feet, still stunned that they had said yes.

"Trust us son," Professor Wagner had said. "We've had stranger requests. Just bring back good hard data. That's what the bean-counters like."

And there lay the problem in a nutshell.

How do I collect data on the absence of something?

It wouldn't be for want of trying. He was armed with cameras, EMF meters and as much computing power as he was able to carry around with him. Now all he had to do was wait, and trust in his theory.

As he lay in his tent that night he pondered the wisdom of even being here. Yes, it might prove his theory. But there was also a chance, a very real chance, that he too would become one of the *disappeared*, just another name on a list of unsolved vanishings. It was a chance he was willing to take, for if he was right, he was on the verge of solving some of the great mysteries, from the missing Roman legion all the way to the *Marie Celeste*. Fortune and glory might wait just around the corner.

As night fell he daydreamed; of book deals and chat shows, of front-page headlines and TV appearances. He found that he was too energized to sleep and eventually, after an hour of tossing and turning in his sleeping bag, he got up and checked over some of the cases that had brought him to this place. He spent the next few hours studying the apocryphal tales surrounding the Flannan lighthouse in Scotland, and the supposed disappearance of three of the keepers in 1900 — another dot on his map.

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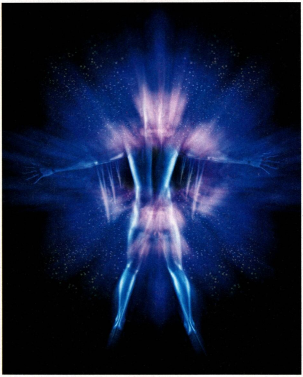

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He'd worked for many long hours on the map, approaching it from many angles, looking for a pattern, a rhythm that might correlate the disappearances with some physical aspect that could be measured by his instruments. He'd cross-referenced his pattern with changes in the magnetic field, with sunspot activity, with lunar cycles, even with daily fluctuations in the stock market. He only hit the jackpot when he took into account the orbit around the Sun, and the long variations caused by the precession of the equinoxes. He found that there was a certain region of space that, whenever Earth passed through it, caused the now famous disappearances. The scale of the vanishing seemed to depend on how close the planet brushed against this spatial anomaly. And it was that anomaly that Frank hoped to measure over the coming day.

He finally fell asleep with dreams of fortune and glory, and woke at dawn to a sparkling clear morning, and the sound of his meters clicking.

It was happening. It was really happening.

The air tingled, as if suddenly supercharged with static, and the hairs Frank's arms and neck all stood up at once. Reality seemed to slide and slip, as if the very fabric of nature was melting and becoming blurred. Frank struggled to focus on his equipment, but that too seemed to blur and fade, so much so that no meter readings were possible.

Just bring back good hard data. That's what the bean-counters like.

Frank wailed. His dream of glory was fading fast, and without data all would be for nothing. He scrambled among the equipment, desperate to reach his laptop. He was so preoccupied that he failed to notice that the world outside his tent had gone.

The laptop went away just as he put a hand on it. Blue static ran across his skin and crackled like damp sticks in a fire.

Frank blinked. In that time, he too went away.

He floated, in darkness filled with swirling fog containing occasional flashes of blue lightning. There was no sound, but there was a profound feeling of being at rest, of being *coddled* by the thick fog.

There was no panic, and Frank's curiosity overcame any sense of fear. He examined the timing of the lightning, hoping once again to discern a pattern, a rhythm that he might identify and use to find a way out of his predicament.

The fog started to part and clear. A blue haze hung ahead of him, and Frank drifted towards it, carried by an unseen tide. He was eagerly awaiting what would come next.

Right up until the moment when the blue haze parted and a vast mouth filled with banks of razor teeth opened up.

He realized his main mistake even as the jaws clamped shut at his waist.

There was one rhythm common throughout nature that he'd failed to take into account.

Feeding time. ■

William Meikle *is a Scottish writer resident in Canada with 10 novels published in the genre press and more than 200 short-story credits in 13 countries.*

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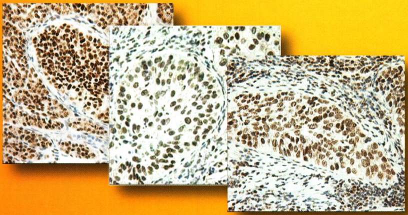

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